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Development of a novel molecular assay for the ultra-rapid diagnosis of acute promyelocytic leukemia by RT-Q-LAMP technology

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Il lavoro presentato in questa tesi è strettamente confidenziale, per questo motivo è stata presentata la domanda di embargo, della durata di 3 anni dal conseguimento del titolo (p.114).

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RIASSUNTO

Lo scopo di questo progetto di dottorato è lo sviluppo di un saggio molecolare ultrarapido per la diagnosi della Leucemia Promielocitica Acuta (LAP) basato sulla tecnologia molecolare LAMP (Loop mediated isothermal AMPlification), metodica non-PCR di amplificazione di acidi nucleici, molto rapida e altamente specifica.

La leucemia promielocitica acuta, nonostante rappresenti il 15-18% dei casi di leucemia mieloide acuta, è caratterizzata da una progressione della malattia particolarmente aggressiva. La sua peculiarità è la presenza alla diagnosi, nell'80% dei casi, di una severa sindrome emorragica. Questa disfunzione della coagulazione può portare alla formazione di trombi, ma più spesso si manifesta sotto forma di emorragie cerebrali che, prima dell'avvento della rivoluzionaria terapia, causavano la morte precoce di quasi il 10% dei pazienti.

La mutazione avviene a livello di un progenitore mieloide, il promielocita, che prolifera in modo incontrollato, rimanendo in uno stato non differenziato accumulandosi nel midollo e in circolo. I promielociti contengono numerose sostanze pro-coagulanti che, in caso di rottura della cellula, possono causare l'attivazione della cascata della coagulazione che, portando alla formazione di trombi e gravi emorragie, causa la morte precoce nei pazienti non trattati.

Per il trattamento di questa leucemia è disponibile un farmaco salva-vita, l'ATRA (All-Trans Retinoic Acid). L'ATRA induce la differenziazione dei promielociti, prevenendo la loro eventuale rottura e la liberazione delle sostanze pro-coagulanti e quindi riducendo in modo drastico gli episodi di morte precoce.

A livello genetico la malattia è causata nel 99% dei casi da una traslocazione bilanciata tra cromosoma 15 e 17, che porta alla formazione della proteina chimerica PML-RARα. Nel gene RARA il breakpoint avviene costantemente nell'introne 2, mentre è variabile nel gene PML, dando luogo a tre diverse isoforme:

- bcr1, in cui il breakpoint si trova tra esone 6 ed esone 7 (forma Long), presente nel 55% dei pazienti;
- bcr3, in cui il breakpoint si trova tra esone 3 ed esone 4 (forma Short), presente nel 40% dei pazienti;
- bcr2, in cui il breakpoint è variabile e si trova all'interno dell'esone 6, perciò dà origine a trascritti di diversa lunghezza (forma Variable), presente nel 5% dei pazienti.

A causa della veloce e drammatica progressione della malattia e per sfruttare la disponibilità del farmaco salva-vita è necessario avere un metodo di diagnosi molto rapido.

Attualmente la diagnosi si basa sull'analisi morfologica delle cellule del sangue e del midollo, che può fornire una prima indicazione di sospetto di

LAP. Tuttavia questo metodo è estremamente soggettivo e spesso la prima diagnosi morfologica si rivela sbagliata. Per questo motivo le linee guida internazionali impongono la conferma della diagnosi tramite tecniche di citogenetica e di biologia molecolare.

Il test molecolare è basato su una reazione di PCR che riesce a distinguere tra le isoforme bcr3 e bcr1-2. Questa è una procedura che comprende numerosi passaggi: retrotrascrizione, 3 reazioni di PCR (bcr1-2, bcr3 e RARα) e controllo della reazione su gel d'agarosio. La durata totale della procedura può raggiungere le 5 ore, con l'utilizzo di un notevole numero di provette. Per una patologia dal decorso così rapido, causato dalle gravi complicazioni date dalla coagulopatia frequentemente presente alla diagnosi, un tardivo raggiungimento del risultato diagnostico può significare la morte precoce del paziente. Inoltre la PCR necessita di personale specializzato e attento, per evitare possibili cross-contaminazioni, dovute all'alto numero di passaggi e provette, e per la corretta interpretazione delle bande su gel d'agarosio.

Successivamente, per il monitoraggio del paziente, viene eseguita l'analisi quantitativa, tramite RQ-PCR specifica per l'isoforma identificata nella prima fase.

Data l'urgenza della diagnosi, nel 1997 è stata sviluppata dal Dott. Falini una tecnica per la diagnosi di LAP basata sul riconoscimento in immunofluorescenza di una peculiare distribuzione della proteina PML. Questa tecnica è piuttosto rapida (circa 2 ore) e non necessita di estrazione di acidi nucleici. Tuttavia è fortemente soggetta all'interpretazione dei

risultati da parte del personale e non è in grado di distinguere fra le tre isoforme, caratteristica fondamentale per permettere il monitoraggio del paziente durante e dopo il trattamento. Nonostante il notevole vantaggio rappresentato dalla rapidità di questo metodo, le linee guida rendono ancora mandatoria la conferma del risultato con una diagnosi molecolare.

La disponibilità di una tecnologia come LAMP si adatta perfettamente alle necessità cliniche di una rapida diagnosi molecolare di LAP e di una corretta assegnazione delle isoforme per la successiva analisi quantitativa per il monitoraggio del paziente.

LAMP è una tecnologia piuttosto recente, sviluppata in Giappone, inizialmente basata su una tecnica di rilevazione in turbidimetria. Ciò che rende LAMP diversa da PCR è l'amplificazione in condizioni isoterme. Grazie all'utilizzo di una polimerasi con attività strand displacement è possibile eliminare lo step di denaturazione, permettendo la continua amplificazione del templato con una notevole riduzione dei tempi di reazione.

In LAMP si utilizzano 4 primer, che riconoscono 6 regioni diverse e da questo dipende anche la sua elevata specificità. I primer più esterni sono F3 (Forward) e B3 (Backward), che consentono all'enzima di effettuare lo strand displacement nelle fasi iniziali, i primer più interni sono FIP (Forward Inner Primer) e BIP (Backward Inner Primer), composti da due porzioni (F1 ed F2; B1 e B2), complementari una alla sequenza senso e l'altra all'antisenso. Questa caratteristica consente il successivo self-annealing del

singolo filamento e la formazione dei caratteristici loop. Per aumentare la velocità di reazione possono essere inseriti due ulteriori primer, LF ed LB (loop primers), che si legano ai loop che vengono formati grazie al self-annealing.

Nei laboratori DiaSorin la tecnologia LAMP è stata ulteriormente implementata, utilizzando un intercalante fluorescente del DNA, che permette il monitoraggio in real-time dell'amplificazione, ma non del tipo di trascritto. Questo necessita di uno step successivo di analisi di annealing.

Nel presente lavoro di tesi è stato introdotto un ulteriore metodo di rilevazione del segnale di amplificazione, basato sull'utilizzo di sonde fluorescenti. Questa novità ha permesso la rilevazione in real-time di diversi target in reazioni in formato multiplex. Grazie all'utilizzo dello strumento Liaison IAM, sviluppato da DiaSorin, che permette la rilevazione di un segnale di fluorescenza in tre colori, è possibile discriminare fino a tre target diversi in ogni reazione, chiaramente identificati grazie al software associato allo strumento. Per i tre trascritti sono state dunque disegnate tre sonde coniugate con un fluoroforo diverso, che potessero essere lette in modo specifico nei tre canali.

Questo metodo di rilevazione ha permesso una vera e propria detection in real-time, in quanto non è più necessario attendere lo step di annealing per distinguere i vari trascritti.

Un ulteriore punto di miglioramento è stata l'introduzione di una polimerasi ingegnerizzata in grado di effettuare la retrotrascrizione dell'RNA in un unico step e in un'unica provetta.

Per ogni trascritto e per il controllo interno è stato selezionato un set di primer specifico e rapido, comprendente 5 primer e una sonda marcata.

Per consentire la rilevazione dei due trascritti più frequenti (bcr1 e bcr3), è stata sviluppata una reazione in triplex, comprendente anche un controllo interno di reazione. Per completare il pannello è stata sviluppata anche la reazione in duplex per l'amplificazione di bcr2, anch'essa comprendente il controllo interno.

Il controllo interno prescelto è stato il gene housekeeping GUSβ. Rispetto al controllo esterno utilizzato in PCR, la presenza di un controllo interno di reazione è un forte vantaggio. Questo infatti permette la validazione dei risultati negativi e l'individuazione della presenza di sostanze interferenti con la reazione. Inoltre permette di valutare la qualità dell'RNA utilizzato.

Una volta identificata la formulazione finale, è stato sviluppato un protocollo di liofilizzazione in modo da poter fornire i reagenti in un formato che permetta la rapida e facile esecuzione del test.

Le reazioni sono state inizialmente testate su plasmidi sintetici contenenti le sequenze delle traslocazioni.

Sui due saggi sono state valutate specificità (assenza di dimeri di primer e di amplificazione in RNA che non portano la traslocazione di interesse) e

sensibilità (minima dose di target rilevabile nel 95% dei casi). Per la traslocazione bcr1 è stato possibile testare la sensibilità su RNA estratto dalla linea cellulare NB4, caratterizzata dalla presenza della mutazione bcr1. Per le traslocazioni bcr2 e bcr3, per le quali non è disponibile una linea cellulare, la sensibilità è stata valutata su basse copie di plasmide, diluite in RNA della linea cellulare HL60.

Entrambi i saggi si sono dimostrati estremamente specifici e sensibili. Nessuna amplificazione aspecifica è stata rilevata nei 40 minuti di reazione e il livello di sensibilità raggiunta (RNA NB4 diluito 1:1000 in RNA di HL-60 per bcr1; 10 copie di plasmide per bcr2; 30 copie di plasmide per bcr3) si è dimostrato estremamente adeguato alle richieste cliniche per un test rivolto alla diagnosi di pazienti leucemici all'esordio.

Sui due saggi è stato eseguito un approfondito studio di robustezza, andando a valutare le capacità di amplificazione anche in condizioni estreme quali l'utilizzo di bassissime quantità di RNA (25 ng/reazione) o di RNA parzialmente o altamente degradato. In ogni condizione i saggi si sono dimostrati estremamente robusti, permettendo sempre la validazione del risultato grazie all'amplificazione del controllo interno.

La validazione finale delle performance è avvenuta su campioni clinici negativi e di pazienti positivi per le tre traslocazioni, diagnosticati tramite PCR presso i reparti di Ematologia dell'Azienda Ospedaliera Papa Giovanni XXIII e del Policlinico Tor Vergata, due dei più importanti centri di riferimento per i casi di LAP.

I saggi sono stati validati su 91 campioni positivi (43 bcr1, 11 bcr2, 37 bcr3) e su 105 campioni negativi (54 donatori sani, 51 pazienti affetti da altre patologie) la cui estrazione è stata effettuata a partire da midollo o sangue periferico con diversi metodi (Qiagen RNeasy Mini Kit, fenolo-cloroformio e Maxwell 16 Instrument).

Tutti i campioni di RNA positivi sono stati correttamente amplificati dai saggi RT-Q-LAMP in circa 15 minuti. Nei campioni negativi il controllo interno si è correttamente amplificato intorno ai 25 minuti, su un tempo totale di reazione di 40 minuti, contro le 5 ore impiegate da PCR.

Per 7 casi dubbi, in cui non era stato possibile determinare con certezza se il trascritto fosse bcr1 o bcr2 a causa dell'ambiguità della grandezza del prodotto di PCR, RT-Q-LAMP è stata in grado di assegnare in modo accurato il trascritto corretto, anche grazie al risultato oggettivo fornito dal software integrato nello strumento Liaison IAM.

All'interno del gruppo di campioni positivi si è riscontrata anche la presenza di alcuni casi particolari, successivamente sequenziati, che RT-Q-LAMP è sempre stata in grado di amplificare, dimostrando la sua efficienza anche in casi di traslocazioni inconsuete.

Concludendo, questo lavoro di tesi riporta lo sviluppo di un prodotto basato sulla tecnologia RT-Q-LAMP ultrarapido per la diagnosi di LAP, in grado di rispondere al forte bisogno clinico di una tempestiva diagnosi molecolare per prevenire, grazie alla somministrazione di un farmaco salva-vita, le gravi conseguenze della coagulopatia associata alla malattia, principale causa di morte precoce nei pazienti non trattati.

Sono stati sviluppati due saggi ultrarapidi e di semplice esecuzione che, partendo direttamente da RNA, sono in grado di identificare e discriminare in 15 minuti i tre trascritti bcr1, bcr2 e bcr3, insieme ad un controllo interno di reazione per validare i risultati negativi. Sensibilità e specificità dei saggi sono state valutate su plasmidi sintetici e validate su campioni clinici positivi e negativi, dando in entrambi i casi ottimi risultati. Entrambi i saggi si sono poi rivelati molto robusti anche se utilizzati su campioni di RNA non ottimali.

Rispetto ai metodi attualmente utilizzati, come la PCR, dispendiosi in termini di tempo, reagenti e che necessitano di personale specializzato, questi nuovi saggi RT-Q-LAMP, grazie alle caratteristiche sopracitate, si dimostrano estremamente competitivi e perfetti nel rispondere ai reali bisogni clinici.

INTRODUCTION

1. ACUTE PROMYELOCYTIC LEUKEMIA (APL)

Acute Promyelocyitic Leukemia (APL) has been described for the first time in the late 1950's as a distinct entity among Acute Myeloyd Leukemias (AMLs). It represents about 15-18% of AMLs. There is not a higher incidence of the disease linked to gender, but APL affects in particular patients between 15 and 60 years of age, a lower range compared to AMLs which are more frequent in older patients (> 60 years old)[1].

Its characterization was possible thanks to the peculiar morphology, which presents an excess of abnormal hypergranular promyelocytes in bone marrow and peripheral blood[2].

APL was considered, until late 1980s, as one of the most rapidly fatal tumours, with the majority of patients dying for haemorrhages within 10 days from the presentation of symptoms. However the advent of differentiation therapy with ATRA (*all-trans* Retinoic Acid)[3, 4, 5], made possible by the cloning of the fusion protein[6, 7, 8], has radically changed the outcome of this disease, dramatically reducing the number of early deaths[2].

1.1 Clinical features

Unlike other AMLs, APL is generally characterized at diagnosis by low leukocyte counts and blast infiltration of bone marrow (BM) only. An exception is the particular variant M3v in which hyperleukocytosis is usually

found[9].

Also epidemiologically APL differs from the other myeloid leukemias since it occurs most frequently between 15 and 60 years of age[9]. One study reported also prevalence of cases in spring and autumn as compared to winter and summer[1].

At presentation APL is characterized by the presence in 80-90% of the patients of a severe haemorrhagic syndrome[10]. The most involved sites are brain and lungs, leading to fatal hemorrhage in up to 20% of patients, before the introduction of ATRA. Cerebral hemorrhage is not only dangerous as cause of early death, but also because it can potentially seed later central nervous system relapse[2].

The pathogenesis of the coagulopathy in APL seems to be linked to activation of the clotting system, increased fibrinolytic activity and non-specific proteinase activity. These effects depend on the leukemic clone itself, which overexpresses different molecules[11]:

- tumor-associated procoagulants (Tissue Factor, **TF** and Cancer Procoagulant, **CP**)[12],
- **annexin-II**, which promotes primary fibrinolysis[13],
- granulocytic proteases, such as **elastase** and **chymotrypsin**, present in the granules of the promyelocites, which promote proteolysis of clotting factors and fibrinogen[14].

Thrombotic complications, in many cases fatal, have also been reported at both arterial and venous level, but this feature is less representative of

APL, being common to many types of acute leukemias[9].

1.2 Molecular pathogenesis

The diagnostic hallmark of APL is a balanced reciprocal translocation between the long arms of chromosomes 15 and 17 [t(15;17)(q22;q21)], leading to fusion of the genes encoding ProMyelocytic Leukemia protein (PML) and Retinoic Acid Receptor Alpha (RARA) to generate the PML-RARα oncoprotein. Variant chromosomal translocations (eg. t(11;17) PLZF-RARA and t(5;17) NPM-RARA) can be detected in approximately 1% of APL patients[15].

On chromosome 17 the breakpoints are constantly localized within a 15 kb DNA fragment of the RARA intron 2. By contrast, three regions of the PML locus are involved in the translocation breakpoints giving rise to three different transcripts (Figure 1):

- **bcr1**, with the breakpoint located in intron 6, representing about 55% of cases
- **bcr2**, in which the breakpoint occurs in exon 6, in only 5% of patients
- **bcr3**, with the breakpoint located in intron 3, representing about 40% of cases[16].

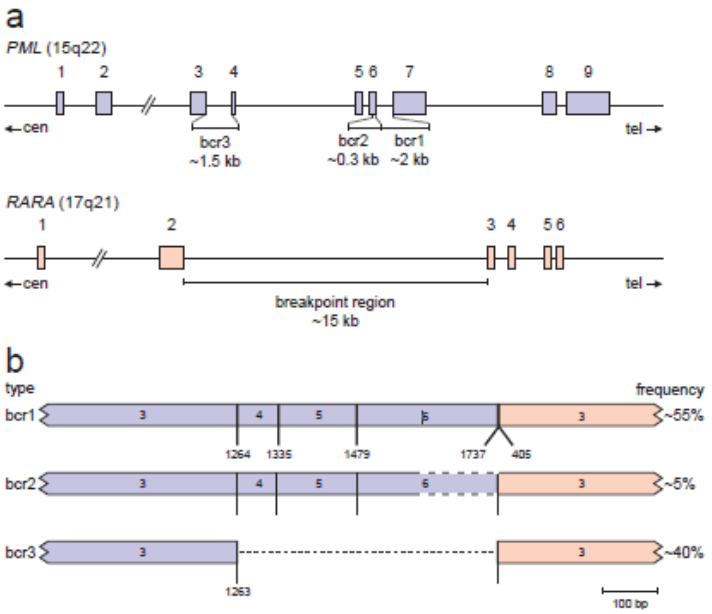

Figure 1. (a) Schematic diagram of the exon/intron structure of the PML and RARA genes, involved in t(15;17)(q22;q21)**.** (b) Schematic diagram of the three types of PML-RARA transcripts, related to the different PML breakpoint regions. The numbers under the fusion gene transcripts refer to the first nucleotide of the involved exon[16].

1.3 The role of PML-RARα

The chimeric protein PML-RARα, resulting from the transcription and translation of the translocated DNA, retains the DNA-binding and ligand-binding domains of RARα and the multimerisation domain of PML. In normal cells PML is a main constituent of nuclear bodies, which are matrix-associated multiprotein-containing domains involved in various biological functions like DNA-damage response and microorganism resistance through regulation of a wide range of proteins, among which are various

transcription factors[17]. In contrast, in APL, the expression of PML-RARα disrupts the localisation of the wild-type PML from nuclear bodies to numerous micro speckles and induces a maturation block at the promyelocytic level[18].

Various mechanisms have been proposed for PML-RARα functioning. It has been suggested that it can form homodimers without RXR[19] or that it forms PML-RARα oligomers that heterodimerise with RXR. RXR is the partner for the wild type RARα in recruiting co-repressors and silencing gene expression[20, 21, 22]. In physiological conditions the complex RARα-RXR undergoes a conformational change after the binding of ATRA to RARα, which allows the recruitment of gene-activating complexes. In APL, PML-RARα has lost the potential to respond to fluctuations in physiological ATRA concentration, and as a result acts as a constitutive transcriptional repressor for many genes through binding to Retinoic Acid Responsive Elements (RAREs)[18].

In addition, it has been suggested that during transformation PML–RARα induces a multitude of alterations in the chromatin architecture, achieved through the recruitment of various epigenetic-modifying factors, like histone deacetylase and DNA methyltransferases[23, 24].

Several studies attempted to correlate the type of PML-RARA transcript either with clinico-biologic features at diagnosis or with treatment response and outcome, with no conclusive results[25, 26, 27, 28].

At diagnosis no correlations have been found for the 3 isoforms with respect to sex, platelet count, presence of coagulopathy or retinoic syndrome. However, patients with bcr3 transcript can show significantly higher white blood cell counts and more frequently M3v morphology. Although bcr3 transcript correlates with these adverse prognostic features, this association does not translate into poorer outcome as compared to patients with bcr1 or bcr2 transcripts, in the context of combined ATRA and chemotherapy regimens.

in vitro studies have shown significant differences in retinoid binding and transcriptional activation properties of bcr1 and bcr3 PML-RARα isoforms[29] and a trend toward less favourable prognosis is reported for patients with the bcr3 isoform[25, 30, 31, 32]. This can be due to the higher frequency of bcr3 in the M3v variant, which is more aggressive. However, no significant correlation has been found between different isoforms and overall survival or complete remission.

1.4 Diagnosis

Although the characteristic morphological features can lead to identification of many APL cases, a definitive diagnosis of APL is possible only through cytogenetic and molecular studies[10].

The identification of the specific translocation has a fundamental implication for the success of the treatment, indeed the front-line approach is different from that used in other AMLs, and is effective also in controlling the life-threatening coagulopathy[2].

Although morphologic diagnosis is straightforward in the majority of hypergranular cases, it appears insufficient for the identification of each and every patient who would benefit from ATRA-containing treatments. In fact, ATRA is effective in leukemia cells expressing only the PML-RARα protein, whereas cases with cytological features of APL but bearing variant translocations, such as the t(11;17) expressing the PLZF/RARα fusion, are ATRA-unresponsive[33, 34, 35]. Vice versa, the APL microgranular variant, which expresses PML-RARα and is ATRA-sensitive[33, 34], is, on morphological grounds, hardly distinguishable from other AMLs or myelomonocytic leukemias[36].

Actually, the diagnostic *iter* starts from morphologic analysis of the blood smear, followed by the molecular confirmation through cytogenetics and RT-PCR. Some specialized laboratories also use immunofluorescence for the detection of the chimeric protein.

1.4.1 Morphological analysis

In the FAB (French-American-British) classification, APL is categorized as an M3 subtype which typically presents pancytopenia, but about a quarter of the patients can present a microgranular variant[37, 38], characterized by an elevated white blood cell count. A rare basophilic variant has also been described (Figure 2D)[39, 40].

The characteristic leukemic cells in the classical hypergranular APL are abnormal promyelocytes with an abundant cytoplasm filled with large granules (Figure 2A). The cytoplasm may be so densely packed with granules that nuclear details are obscured. The nuclear contour is irregular,

and in some cells the nuclei have a bilobed appearance. Auer bodies are commonly found in many cells, and sometimes they are regrouped in bundles to form the so-called faggots (Figure 2A and B). These typical hypergranular leukemic cells are mostly present in the bone marrow and less evident in the peripheral blood. The microgranular variant is characterized by the presence of leukemic cells with very minute granules in the cytoplasm that are not easily perceptible on light microscopy (Figure 2C)[10].

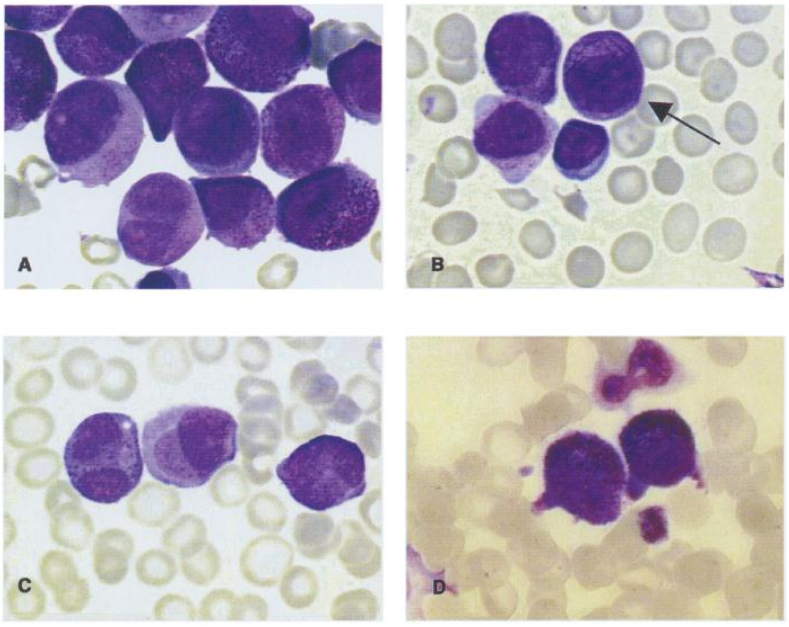

Figure 2. Morphologic analysis of patients affected by APL. (A) Classical APL with numerous hypergranular promyelocytes and a cell in the middle containing numerous Auer rods (faggots). (B) A cell with numerous Auer rods (faggots). (C) Microgranular APL with bilobed nuclei. (D) Basophilic variant of APL[10].

1.4.2 Cytogenetic analysis

The cytogenetic analysis is carried out through conventional karyotyping or FISH, employing a probe for PML and a probe for RARA. Conventional cytogenetics can detect up to 90% of APL cases with a PML-RARA rearrangement, but is limited in its ability to detect cryptic translocations[41]. FISH can be accomplished in a shorter time frame and can detect a PML-RARA fusion with a very high sensitivity and specificity, including some cryptic translocations[41]. However, a few cases of PML-RARA rearrangements have been reported to be detectable only by PCR or other molecular techniques because of submicroscopic insertions beyond the limit of resolution of FISH [42, 43].

Despite the relatively low diagnostic sensitivity of cytogenetics, it retains an important role and should always complement, and not be a substitute for, molecular diagnostics. In fact, chromosome partners of 17q other than 15q, complex translocations involving more than two chromosomes, and additional abnormalities besides the t(15;17) are only detectable by cytogenetic studies. Their identification provides potentially relevant clinical information, in particular in the case ATRA-resistant APL variants[2].

1.4.3 Immunofluorescence

Thanks to the biological features of the cells carrying the PML-RARα chimeric protein, it has been possible to develop a technique based on the use of fluorescent antibodies[44].

This test exploits the differential localization of the wild type PML protein and mutated PML-RARα. Immunolabelling of the wild-type PML protein

with the PG-M3 monoclonal antibody (MoAb) directed against the amino terminal portion of the human PML protein, produces a characteristic nuclear speckled pattern that is due to localization of the protein into discrete dots (5 to 20 per nucleus), named PML nuclear bodies (Figure 3, single arrow)[45, 46]. The architecture of these nuclear domains is disrupted in APL cells bearing the t(15; 17), resulting in the loss of the speckled pattern and its substitution with many, small nuclear dots (Figure 3, double arrow)[47, 48].

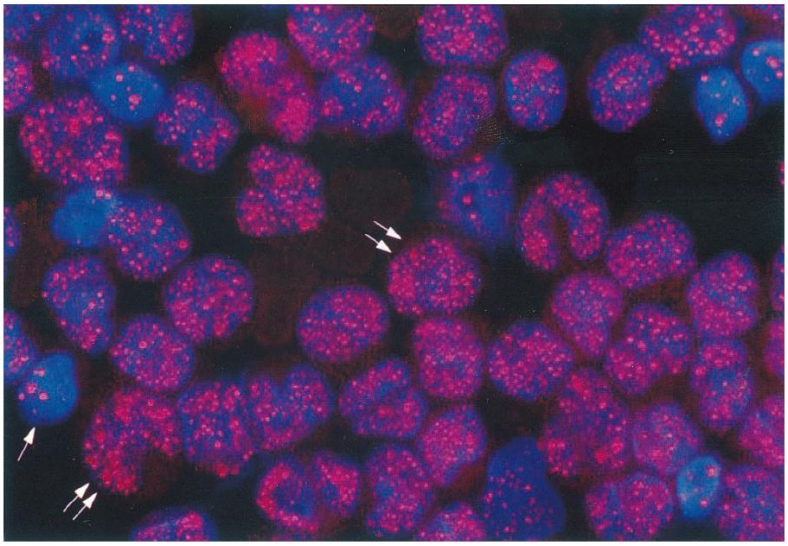

Figure 3. Immunolabeling of a microgranular APL case with rodhamine-labelled PG-M3 antibody. The nuclei of APL cells (double arrows), stained with DAPI, contain many tiny dots (microgranular pattern). The single arrow points to a residual normal hematopoietic cell showing the speckled positivity of wild-type PML [44].

The study of the PML distribution pattern in leukemic cells provides a rapid (2 hours) diagnostic approach for APL, since it does not require RNA extraction. However, its specificity for the detection of PML protein does not allow the identification of translocations not involving chromosome 15. Moreover, since the approach detects the PML protein, cannot provide the specific isoform, bcr1, bcr2 or bcr3, necessary for the monitoring of the patient status.

1.4.4 RT-PCR

RT-PCR is mandatory for the diagnosis of APL, mainly due to the ability to determine the PML breakpoint that allows the definition of the correct strategy for the following minimal residual disease monitoring.

High-quality RNA and efficient RT are the crucial determinants for successful RT-PCR of PML-RARA. Because of frequent leukopenia and the associated coagulopathy, the yield and quality of RNA from diagnostic samples are frequently poor[49, 50].

A specific and standardized protocol has been developed by a laboratory network for the detection of PML-RARA with RT-PCR. The standardization and quality control not only concerns the primers to be used, but also the RT reaction and the PCR protocol as well as the usage of common positive and negative controls[16]. Following this protocol, RT-PCR allows the identification of the translocation in about 4-6 hours, excluding the extraction step.

Two RT-PCRs have been developed for the detection of bcr1/2 and bcr3 respectively. This does not allow a direct discrimination between bcr1 and

bcr2, made possible only by the interpretation of the length of the PCR fragment on the gel. As a consequence, a correct discrimination of the right transcript is not possible in the majority of the bcr1/2 cases, since bcr2 transcript has variable length that can be very close to the bcr1 transcript.

The declared sensitivity of the method on RNA obtained from APL patients as well as with RNA from the NB4 (bcr1) cell line is[16]:

- for the single step PCR, 10^{-2} on bcr1, bcr2 and bcr3 patient's RNA; 10^{-3} on NB4 (bcr1) cell line RNA
- for the nested PCR, 10^{-3} on bcr1, bcr2 and bcr3 patient's RNA; 10^{-4} on NB4 (bcr1) cell line RNA

1.5 Monitoring of residual disease

In the last decade, the availability of differentiation therapy with ATRA and ATO has produced a remarkable improvement in the outcome of patients with APL, with the majority of them going into complete remission. The monitoring of the amount of transcript is however very important in order to identify the relatively small subgroup of patients at risk of relapse[51, 52, 53].

Overall, there is general agreement that a positive PML-RARA test after consolidation is a strong predictor of subsequent hematological relapse, whereas repeatedly negative results are associated with long-term survival in the majority of patients [51, 52, 53].

Also for the monitoring of the residual disease a standardized protocol has been taken in place, using real-time quantitative PCR (RQ-PCR), which

allows the quantification of the transcript and therefore can provide the kinetics of the amount of transcript at different time points. Also in this case the standardization involves the RT step, design of primers, real-time PCR protocol and results analysis. Three different real-time PCRs have been developed, one for each transcript that needs to be previously discriminated with RT-PCR. The quantification is calculated relatively to a housekeeping gene, more frequently ABL, encoding for Abelson tyrosine kinase or GUSB, encoding for beta glucoronidase [54]. However many laboratories use RT-PCR as well for the monitoring of the disease.

1.6 Treatment of APL

As for other type of cancer, chemotherapy has been used as first approach to treatment. In 1973, Bernard et al[55] demonstrated that APL leukemic cells were relatively sensitive to chemotherapy, that yielded a complete remission rate of 55% in 34 patients with APL. From then on, chemotherapy composed of an anthracycline (daunorubicin, idarubicin, or others) and cytosine arabinoside (Ara-C) has been the frontline treatment of APL, making possible to reach CR (Complete Remission) rates of 75% to 80% in newly diagnosed patients[56, 57]. Despite such progress, the median duration of remission ranged from 11 to 25 months and only 35% to 45% of the patients could be cured[58].

In 1985, the introduction of all-trans retinoic acid (ATRA) opened a new page in the history of APL treatment: the differentiation therapy. Optimization of the ATRA-based regimens combining ATRA and

chemotherapy has further raised the complete remission rate up to 90% to 95%, and a 6-year disease-free survival up to 86%. The application of arsenic trioxide (ATO) since the early 1990s has further improved the clinical outcome of refractory or relapsed as well as newly diagnosed APL. Moreover, a deeper reduction in PML-RARα transcript and longer survival in newly diagnosed APL have been achieved when ATRA was combined with ATO compared with therapy with ATRA or ATO alone[18].

Risk stratification is very important in the treatment of APL patients, as those with low-risk disease (white blood cell count (WBC) ≤ 10.000/µl) are generally treated with less intensive regimens than those patients presenting with high-risk disease (WBC > 10.000/µl). In the past two decades, therapy for newly diagnosed APL has evolved from ATRA plus chemotherapy backbone for all patients, to the addition of ATO, to ATRA with omission of chemotherapy in low-risk patients as a new standard of care [59].

As supportive measure to the treatment with ATRA, the transfusion of platelets can be employed, in order to contrast the effects of coagulopathy, at least until the ATRA shows its action and genetic diagnosis is estabilished[60].

1.6.1 Mechanisms of action of ATRA and ATO in APL differentiation therapy

ATRA is a natural ligand of RARα and can also bind to PML-RARα, but its physiological concentrations cannot induce the conformational change responsible for the interruption of transcriptional block. At pharmacological concentrations (10^{-6}-10^{-7} M), ATRA triggers the conformational change and the relieve on the differentiation block[18, 61].

The effect of ATRA is double, since it also significantly enhances a number of components of the Ubiquitin/Proteasome System (UPS), inducing the degradation of the chimeric protein through the UPS itself[62, 63].

Thanks to these effects, ATRA induces terminal differentiation of leukaemic promyelocytes and leads to an immediate improvement in bleeding symptoms and almost complete resolution of the associated coagulopathy within 1–2 weeks of treatment[60].

ATO is probably the most effective single agent used in the treatment of APL, with a complete remission rate higher than 90% in many studies[64, 65]. It directly binds the PML-RARA oncoprotein inducing its proteosomal degradation leading to apoptosis of leukaemic cells [60].

The therapeutic effect of ATO is driven by three different mechanisms. Under high concentration (1-2 x10^{-6} M) it induces apoptosis while under low concentrations (0.25-0.5 x10^{-6} M) and upon a longer treatment course, ATO tends to promote differentiation of APL cells[66]. Moreover it also

promotes the degradation of the chimeric protein by a significant degree of sumoylation of PML and PML-RARα[67].

All the mechanisms of action of ATRA and ATO synergistically interact when the two drugs are used in combination, leading to a shorter time in achieving complete remission and longer disease free survival.

1.6.2 Prognosis

With the success of ATRA- and ATO-based induction regimens, the prognosis of APL has changed markedly from that of a rapidly fatal acute leukemia, as it was in the 1960s and 1970s, to that of a highly curable disease. Recently, the question emerged as to whether chemotherapy could safely be eliminated or minimized to reduce treatment associated toxicities and long-term complications. With this aim, a multicenter trial comparing ATRA plus idarubicin with ATRA plus ATO was conducted in patients with low- to intermediate-risk APL[68].

The results showed a significant superiority of the ATRA-ATO treatment, compared to the ATRA-chemotherapy:

- the 2-year EFS rates were 97% in the ATRA–ATO group, and 86% in the ATRA–chemotherapy group
- the 2-year OS probability was 99% in the ATRA–ATO group, as compared with 91% in the ATRA–chemotherapy group

2. LOOP-MEDIATED ISOTHERMAL AMPLIFICATION (LAMP)

In 2000 Notomi et al[69] firstly described a new technique for the isothermal amplification of nucleic acids, named Loop-mediated isothermal AMPlification (LAMP).

This method employs a DNA polymerase with strand-displacement activity and a set of 4 primers specifically designed to recognize 6 different regions on the target gene. A couple of primers, the outer primers (F3 and B3), act in the initial phases of the reaction, and the other couple, the inner primers (FIP and BIP), promotes the reaction process.

FIP and BIP (Forward/Backward Inner Primer) contain two distinct sequences corresponding to the sense (F2 and B2) and antisense (F1c and B1c) sequences of the target DNA, one for priming in the first stage and the other for self-priming and loop formation in later stages.

The mechanism of LAMP amplification reaction includes 3 steps: production of the starting structure, amplification and elongation, and recycling.

Inner primer FIP hybridizes to F2c in the target DNA and initiates complementary strand synthesis (structures 1-2). Outer primer F3 slowly hybridizes to F3c in the target DNA and initiates strand displacement DNA synthesis, releasing a FIP-linked complementary strand, which can form a looped out structure at one end (structure 4). This single-stranded DNA serves as template for BIP-initiated DNA synthesis and subsequent B3-primed strand displacement DNA synthesis, leading to the production of a dumb-bell form DNA (structure 6), which is quickly converted to a stem-

loop DNA by self-primed DNA synthesis (structure 7). The dumb-bell serves as the starting structure for LAMP exponential amplification, the second stage of the LAMP reaction. To initiate LAMP cycling, FIP hybridizes to the loop in the stem-loop DNA (structure 7) and primes strand displacement DNA synthesis, generating as intermediate one gapped stem-loop DNA with an additional inverted copy of the target sequence in the stem, and a loop formed at the opposite end (structure 8). Subsequent self-primed strand displacement DNA synthesis yields one complementary structure of the original stem-loop DNA (structure 10) and one gap repaired stem-loop DNA with a stem elongated to twice as long (double copies of the target sequence) and a loop at the opposite end (structure 9). Both these products then serve as template for a BIP-primed strand displacement reaction in the subsequent cycles, the elongation and recycling step. Thus, in LAMP the target sequence is amplified 3-fold every half cycle.

The final products are a mixture of stem–loop DNAs with various stem lengths and cauliflower-like structures with multiple loops formed by annealing between alternately inverted repeats of the target sequence in the same strand.

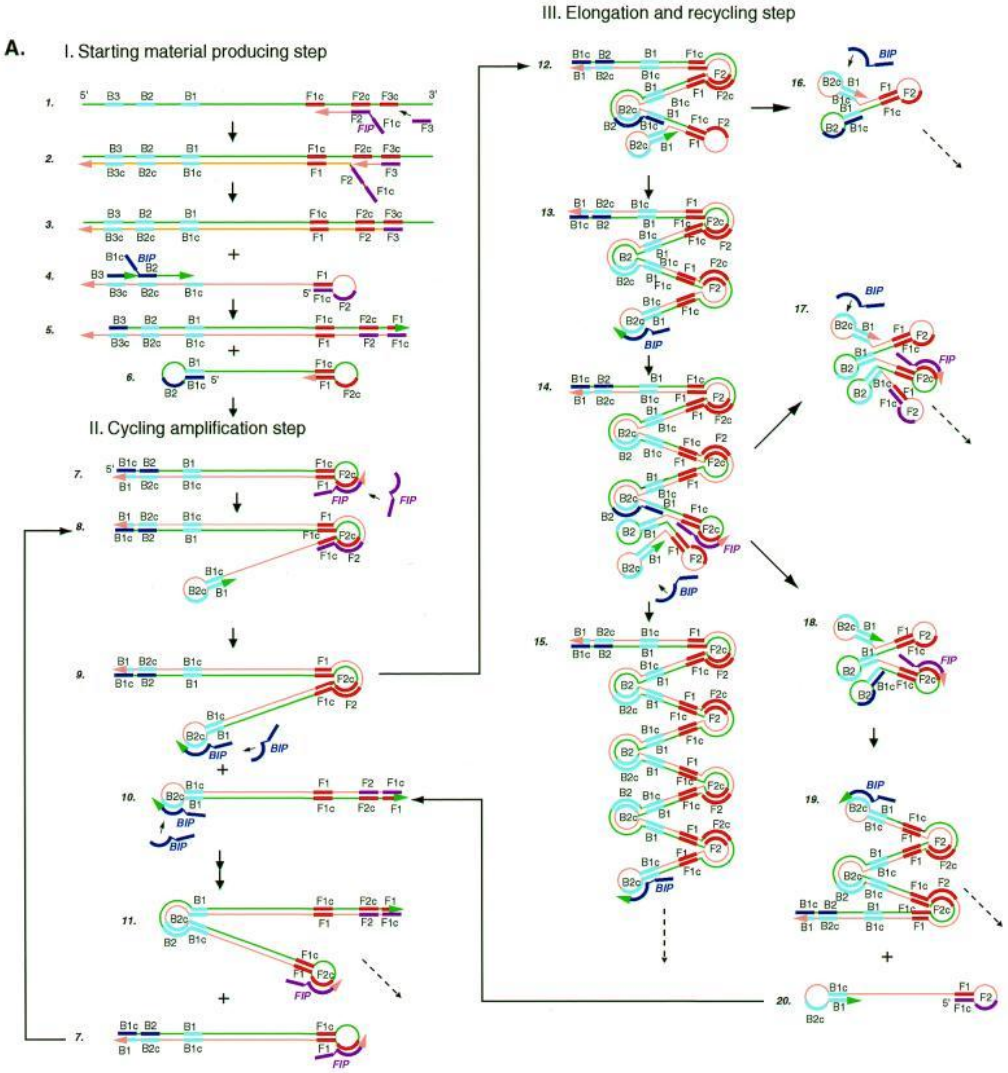

Figure 4. Schematic representation of a LAMP reaction[69].

The use of a strand-displacing DNA polymerase with integrated RT activity allows also the amplification of RNA. In this case the backward primers B3 and BIP act as initiators of the retro-transcription reaction, creating the cDNA molecule used in the following amplification steps.

2.1 Loop primers

Thanks to the strand-displacement activity of the polymerase, LAMP reaction is very fast, but can be even accelerated introducing 2 additional primers, the Loop primers Forward and Backward (LF and LB). Loop primers hybridize to the stem-loops, and prime strand displacement DNA synthesis[70].

The loop primers act as reaction boosters, annealing and extending on available single strand regions on the loops of the dumb-bell structure.

2.2 Amplification detection

Different methods can be used to detect positive LAMP reactions, mainly using turbidimetry and fluorescence, but in recent years also electrochemical methods have been developed.

Turbidimetry detection is made possible by the reaction between pyrophosphate ions, a by-product of polymerization, and magnesium ions, present in the reaction buffer. This reaction produces a white precipitate that can be visualized either with naked eye, at the end of the reaction, or measured in real-time with a turbidimeter[69, 71, 72].

Fluorescence detection is possible by the use of either colorimetric indicators, intercalating dyes or fluorescent probes.

An example of **colorimetric indicator** is calcein, a molecule that binds to manganous ions before the start of amplification. Manganous ions quench calcein green fluorescence, but they are displaced from their binding as LAMP reaction proceeds and a high amount of pyrophosphate ions is produced. In addition, calcein molecules combine with residual magnesium ions, enhancing the green fluorescent signal[73]. This signal can be visualized even at naked eye or irradiating the tubes with a standard transilluminator.

Alternatively, products of LAMP reaction can be directly visualized in real-time using **intercalating dyes**, fluorescent dyes that can bind to the double-stranded DNA as soon as it is synthesized. The isothermal amplification can be visualized by fluorescence detection of the amplicons and an annealing analysis to confirm the product[74].

The most efficient method for the detection in real-time of more than one target is the use of **fluorescent probes**, developed by DiaSorin (Q-LAMP). The use of different fluorophores allow the detection and discrimination in real-time of up to three different targets in multiplex reactions.

2.3 Advantages of LAMP

The primary characteristic of LAMP is the ability to amplify nucleic acids at isothermal conditions, allowing the use of simple and cost-effective reaction equipments.

The use of a polymerase with strand-displacement activity increases exponentially the speed of reaction, unbinding the amplification to the extension step, as instead happens for PCR. This high efficiency of LAMP reaction also determines a deep sensitivity, comparable to that of nested PCR.

The coupling of RT and amplification step, together with the real-time detection, simplifies strongly the diagnostic workup, introducing a one-step method, more reliable and with lower risk of cross-contamination compared to the multi-step procedure of PCR.

Moreover, the multiplex format of RT-Q-LAMP, makes possible the introduction of the internal control, and the specific identification of different targets.

All these characteristics have made LAMP a very useful method for the detection of many different organisms, such as bacteria, viruses, fungi and parasites.

DiaSorin wanted to exploit all these advantages for the detection of translocations and point mutations for the diagnosis of different leukemic diseases.

In particular, in the diagnosis of Acute Promyelocytic Leukemia, this technique can enhance its properties, giving to the laboratories a reliable molecular assay which allows the identification of APL in a very short time, compared to other techniques, potentially saving patients' life thanks to a fast administration of the correct treatment.

MATERIALS AND METHODS

1. cDNA PLASMIDS

For the development of the LAMP assays has been necessary the use of plasmids containing the cDNA sequences of the 3 transcripts and of the internal control. The size of the insertions for bcr1, bcr2 and bcr3 were respectively 787, 754 and 827 bp and 539 bp for GUSβ.

Plasmids have been produced by Life Technologies (GeneArt® Gene Synthesis). The cDNA sequence has been synthetized, cloned into pMA-T vector and transformed into E. Coli. The construct has been purified and sequence-verified.

In order to be sure that our primers will amplify also the highly deleted forms of bcr2 transcript, the shortest variant that has ever been observed in a patient in Azienda Ospedaliera Papa Giovanni XXIII (Bergamo) was chosen to be cloned clone into the plasmid.

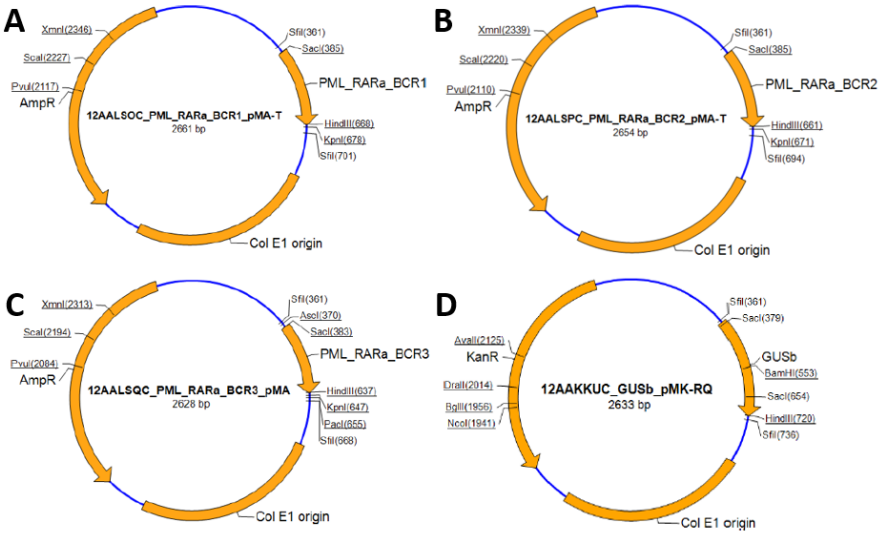

Figure 5. Plasmid's map provided by Life Technologies for bcr1 (A), bcr2 (B), bcr3 (C) and GUSβ (D).

The copy number per µL of each plasmid has been calculated based on the length and on the concentration. Plasmids have been diluted into a buffer containing Tris-HCl and Yeast RNA.

In order to assess the sensitivity, plasmids have been denatured by heating at 95°C for 10 minutes and immediately chilled on ice to prevent re-naturation.

In the final format of the assays the not-denatured plasmids have also been used as positive (bcr1, bcr2 and bcr3) and negative (GUSβ) controls.

2. CELL CULTURE

A stable culture has been established for **NB-4** cell line (t(15;17) (q22;q11-12.1)-DMSZ no. ACC 207), carrying the bcr1 translocation and for negative controls cell lines such as **HL-60** (AML, M2-DMSZ no. ACC 3), **TOM-1** (t(9;22) (q34;q11) p190-DMSZ no. ACC 578), **K-562** (t(9;22) (q34;q11) p210-DMSZ no. ACC 10), **KASUMI-1** (t(8;21) (q22;q22)-DMSZ no. ACC 220), **MV4-11** (t(4;11) (q21;q23)-DMSZ no. ACC 102), **RS4;11** (t(4;11) (q21;q23)-DMSZ no. ACC 508), **REH** (t(12;21) (p13;q22)-DMSZ no. ACC 22) and **697** (t(1;19)(q23;p13)-DMSZ no. ACC 42).

Cell lines NB-4, HL-60, K562 e KCL-22 were plated in medium RPMI 1640 (Gibco Life Tecnologies, Gaithersurg, MD), supplemented with 10% FBS (v/v) (Fetal Bovine Serum) (Lonza, Gaithersurg, MD) and Penicillin-Streptomycin-Amphotericin B Mixture (Lonza). Cell lines 697, Kasumi-1, MV4-11, REH e TOM-1 plated in medium RPMI 1640 (Gibco Life Tecnologies, Gaithersurg, MD), supplemented with 20% FBS (v/v) (Fetal Bovine Serum) (Lonza, Gaithersurg, MD) and Penicillin-Streptomycin-Amphotericin B Mixture (Lonza). Cell line RS4;11 was plated in medium MEMα (Gibco Life Tecnologies, Gaithersurg, MD), supplemented with 10% FBS (v/v) (Fetal Bovine Serum) (Lonza, Gaithersurg, MD) and Penicillin-Streptomycin-Amphotericin B Mixture (Lonza). All the cell lines were incubated at 37 °C in a humidified atmosphere containing 5% CO2.

3. CLINICAL SAMPLES

The final evaluation of relative sensitivity has been performed on 91 positive clinical samples, previously tested with PCR. Relative specificity has been tested on 51 no-APL clinical samples (26 B-CLL, 8 ALL, 13 AML, 2 PV and 2 CML) and 54 healthy donors. These clinical samples have been collected from 1995 to 2014 at two different clinical sites (Bergamo and Rome) from subjects who gave their informed consent.

4. RNA EXTRACTION

Different RNA extraction methods have been used, in order to evaluate their impact on the assays' performances. Since in hospitals the use of automated extractors is increasing, the impact of such extraction on our assays has also been evaluated.

4.1 Qiagen RNeasy MiniKit

Total RNA from cultured cell lines and healthy donors has been extracted by using RNeasy Mini Kit (Qiagen, Hilden, Germany) starting from 10^7 cells lysed in 600 μL of RLT Buffer.

Total RNA from clinical samples has been extracted from mononuclear cells isolated by Ficoll-Hypaque gradient centrifugation and lysed in guanidiniumiso-thiocyanate (GITC).

For the extraction we used the standard protocol described in the RNeasy Mini Handbook (Fourth Edition, June 2012), adding a centrifugation step of 4 minutes at 4000 rpm before the elution step, to increase ethanol removal.

4.2 Phenol-Chloroform

RNA extraction from cultured cells and healthy donors has been performed by using 1 mL of TRIzol® on 10^7 cells. Conversely, clinical sample cells have been resuspended in guanidiniumiso-thiocyanate (GITC).

The extraction has been performed as described by the manufacturer's protocol, with the following modifications:

1. All incubation steps have been performed on ice, instead of room temperature
2. RNA precipitation has been performed in isopropanol overnight at -20˚C, instead of RT for 10 minutes
3. Residual ethanol has been removed by adding a second centrifugation step (1') prior to air drying the pellet on ice.

4.3 Maxwell® 16 Instrument

The evaluation of an automated extraction method has been performed on the Maxwell® 16 Instrument (Promega, Madison, WI, USA).

Total RNA has been extracted from cultured cell lines and clinical samples using Maxwell® 16 LEV simply RNA Blood Kit. This kit provides an efficient extraction based on paramagnetic silica beads and the low elution volumes ensure a highly concentrated RNA.

We started from 12 x 10^6 cultured cells, resuspended in 350µL of Homogenization Solution (Promega) and proceeded with the extraction according to manufacturer's Instructions for Use.

For clinical samples we started from all the available isolated white blood cells treated with 5 mL of the Cell Lysis Solution (Promega) for the destruction of red blood cells. The sample was then centrifuged at 3000 rcf for 10 minutes and the pellet resuspended in 350 µL of Homogenization Solution (Promega) for 10-15 x 10^6 cells, 400 µL of Homogenization Solution (Promega) for more than 15 x 10^6 cells. An equal volume of Lysis Buffer (Promega) and one tenth of the volume of Proteinase K were added to the mixture. The extraction was completed according to manufacturer's Instructions for Use with a final elution volume of 35 µL.

4.4 Extracted RNA quality evaluation

After each extraction the quality of RNA has been evaluated by *Nanodrop 2000* (Thermo Scientific) reading.

The two important parameters which allow the determination of the quality of RNA are A260/A280 and A260/A230 ratio. A260/A280 represents the ratio of the readings at 260 nm and 280 nm and estimates the RNA purity with respect to contaminants that absorb in the UV spectrum, such as proteins. Values between 1.8 and 2.1 indicate highly pure RNA.

The 260/230 ratio estimates the contamination levels of the sample by extraction reagents, such as TRIzol and guanidiniumiso-thiocyanate. Expected values are commonly in the range of 2.0-2.2.

In order to evaluate the integrity of the extracted RNAthe *Bioanalyzer 2100 Instrument* (Agilent Technologies) has been used, with the Agilent RNA 6000 Nano Kit.

Samples have been analysed according to manufacturer's instructions for use. 1 µL of sample (usually 100 ng) was loaded on the chip for the electrophoretic analysis, using the Eukaryote Total RNA Nano II Series assay protocol.

The integrity of RNA has been determined by calculating the ratio between the peaks of 28s and 18s rRNAs, that indicates good RNA quality if around 2. The main instrument to evaluate RNA integrity is the RIN (RNA Integrity Number) software algorithm which allows the classification of total RNA, based on a numbering system from 1 to 10, with 1 being the most degraded and 10 being the most intact.

5. LAMP ASSAYS

5.1 Primer design

A LAMP reaction employs at least 4 primers, which recognize 6 different regions on the target gene. F3 and B3 are the most external primers, which allow the strand-displacement activity of the enzyme at the beginning of the reaction. The inner primers are FIP (Forward Inner Primer) and BIP (Backward Inner Primer), made by 2 parts (F1 and F2; B1 and B2), respectively complementary to the sense and antisense sequence. This characteristic allows the self-annealing of the single strand product and the

formation of the stem-loop structure, which represents the starting point for the LAMP reaction.

In addition, 2 more primers, LF and LB (Loop Forward and Loop Backward), that anneal to the single strand sequence of the loops, can be employed in order to increase the speed of amplification.

In this work a fluorescent probe has been introduced, substituting one of the loop primers, which allows the real time detection of the amplification.

The primer sets for bcr1, bcr2, bcr3 and GUSβ have been initially designed with the software *Primer Explorer*. This free-access software is specifically created to design primers for a LAMP reaction. It is able to design the necessary 4 primers and also the loop primers, using the "nearest neighbour" method for Tm calculation. Among the great number of sets produced in the output we selected the ones in which the forward and backward portions were designed in-between the translocation.

Those sets have been further analysed with the software *Visual OMP* (DNASoftware, Ann Arbor, USA). The characteristic of this software is to provide also predictions of secondary structures, which can help in excluding primer sets that can give primer dimers amplification.

It performs the calculation of Tm by the "nearest neighbour" method, taking also in consideration the different factors influencing the reaction, such as temperature, primers, salts and glycerol concentration.

5.2 Primer sets selection

In silico selection has been based on the absence of primer dimers and non-specific binding to other portions of the target sequence.

Experimental selection has been performed testing each primer set at standard conditions on the specific plasmid (10.000, 1.000, 100 and 10 copies/reaction) to test the speed of amplification and on water samples to test the specificity. The primer sets that passed the selection had at least 98% of specificity and the amplification of the plasmids should be completed within 40 minutes.

5.3 Labelled probes

In the final set, one of the loop primers, the one with the lowest impact on reaction efficiency, has been substituted with a probe that has the same sequence, but cannot be extended and is conjugated with a fluorophore.

For the triplex assay, three different customized fluorophores have been used, specifically read by the channels of the Liaison IAM instrument. For the duplex assay only two of the three fluorophores have been used.

The primers and probes have been synthesized by SGS DNA.

5.4 LAMP reaction – Intercalating Dye

The single reactions for each transcripts have been performed using YO-PRO®-1 Iodide (Life Technologies), an intercalating agent which, once in the double strand helix, emits a fluorescent signal at 509 nm. The fluorescent signal increases proportionally to the amount of amplification product, but

is not specific because can be generated by any double strand product, even primer dimers.

Therefore, in this reaction format is necessary an additional annealing step after the completion of amplification. The annealing analysis is performed through the denaturation of the amplified product at 99°C followed by slow cooling to 85°C. As the temperature decreases, the complementary strands anneal and the intercalating dye is incorporated, increasing the fluorescent signal. The specific annealing temperatures for the three transcript were: 93°C for bcr1, 92°C for bcr2 and 90°C for bcr3.

The LAMP reactions with intercalating dye have been performed on Genie® II Instrument (Optigene) at a constant temperature for 60 minutes.

Each reaction mixture contained the primer set specific for the translocation (F3/B3 0.2 µM, FIP/BIP 1.6 µM, LF/LB 0.8 µM), dNTPs 1.4 mM each, 8 mM $MgSO_4$, Tris-HCl 20 mM, KCl 10 mM, $(NH_4)_2SO_4$10 mM, tween 20 0.1%, YO-PRO®-1 Iodide 1mM, enzyme 0.32 U/µL, 5 µL of plasmid and distilled water (up to the final volume of 25 µL).

5.5 LAMP reaction – Fluorescence

The duplex and triplex reactions have been developed after the introduction of the labelled probes specific for each transcript.

Customized fluorophores have been produced by Cyanagen (Bologna, Italy), with different emission wavelength so that they could be read in three different channels of Liaison IAM instrument without cross-talk effect.

For the triplex reaction bcr1 has been labelled with CHROMIS 530, bcr3 with CHROMIS 570 and GUSβ with CHROMIS 500. In the duplex reaction bcr2 has been labelled with CHROMIS 500 and GUSβ with CHROMIS 530.

CHROMIS 500 (C500) has an absorption wavelength of 496 nm and emits at 506 nm, CHROMIS 530 (C530) has an absorption wavelength of 529 nm and emits at 561 nm, CHROMIS 570 (C570) has an absorption wavelength of 573 nm and emits at 612 nm.

The LAMP reactions with labelled probes have been performed on Liaison IAM instrument (DiaSorin).

Each reaction mixture contained the primer set specific for the translocation (F3/B3 0.2 µM, FIP/BIP 1.6 µM, LF or LB 0.8 µM) and the specific probes 0.8 µM, dNTPs 1.4 mM each, 4 mM $MnCl_2$, Tris-HCl 20 mM, KCl 10 mM, $(NH_4)_2SO_4$10 mM, tween 20 0.1%, enzyme 0.32 U/µL, 5 µL of plasmid and distilled water (up to the final volume of 25 µL).

6. LIAISON IAM INSTRUMENT

The Liaison IAM instrument has been specifically developed by DiaSorin for the execution of the Q-LAMP assays. It performs isothermal amplification and detection of nucleic acids, by real-time measuring of changes in fluorescence in up to three channels.

Liaison IAM detection system consists of three LED for excitation and three photodiodes for detection of three fluorescence channels. 8 optical fibres (one for each well) transmit the light for excitation of the

fluorophores to the wells and consequently conduct the light emitted by the reaction for the elaboration.

The instrument is integrated with a *Liaison IAM Software* that recognises in which channel occurs the amplification and, according to the specific assay performed, is able to return a clear and objective result about the state of the patient (positive or negative) and, if positive, which specific transcript has been identified.

7. DETECTION OF PML-RARA t(15;17) by RT-PCR

The retro-transcription step and the single PCR have been performed as described by the BIOMED standardized RT-PCR protocol[16].

In order to detect all the 3 transcripts 2 different reactions are needed one for the detection of bcr1 and bcr2 and one for the detection of bcr3.

PCR products are run in 1,5% agarose gel and visualized with ethidium-bromide staining[16]. For bcr1 and bcr2 the discrimination of the transcript is based on the length of the PCR product. Sizes of PCR products are listed in Table 1.

	A1 + B	A2 + B
bcr1	381 bp	-
bcr2	345 bp (variable)	-
bcr3	-	376 bp

Table 1. Sizes of PCR product obtained with the two couples of primers. bcr2 length varies according to the position of the breakpoint[16].

A third reaction is separately performed for the amplification of the negative control.

The sensitivity of single PCR reaches 10^{-2} dilution of bcr1 patient RNA in HL60 RNA and is a log more sensitive on NB4 RNA (10^{-3} dilution in HL60 RNA). For bcr3 the single PCR can detect a positive sample's RNA diluted 10^{-3} into HL60 RNA. Using a couple of internal primers to perform the nested PCR this technique can increase sensitivity up to one more log for bcr1 and bcr3[16].

8. FREEZE-DRYING

The freeze drying process has been performed on VirTis AdVantage Plus (SP Scientific) freeze-dryer.

The protocol consists of different phases that have been optimized during the development of the final products:

1. freezing, in which the reaction mix is taken at low temperatures (< -35°C) in order to immobilize the material and define the structure that will be dried;

2. primary drying through sublimation. In this phase vacuum is needed to reach the triple point of water, when the sublimation can occur;
3. secondary drying, in which the temperature increases to 20°C and a further drying of the product is obtained by desorption.

In order to create the solid organized structure that will remain at the end of the process Dextran70 is added to the reagents mix.

The freeze-drying format has been optimized differently for the two assays. The bcr2 duplex assay reaction mix is lyophilized into one single cake, containing dextran plus dNTPs, enzyme, primers and probes.

For the triplex assay, however, it has been necessary the separation in a second cake of the labelled probes that, interacting with the primers, impaired the stability of the freeze-dried reticulum, causing the collapse of the unique cake.

Once freeze-dried, the cakes can be very easily reconstituted adding the two remaining elements of the liquid reaction mix: buffer and manganese.

RESULTS

Two ultrarapid LAMP assays for the detection and discrimination of the PML-RARA transcripts have been developed and tested in terms of analytical specificity and sensitivity. In order to validate their performances, the assays have been tested on clinical positive and negative samples, comparing the results with the RT-PCR reference method.

The assays have been evaluated also in terms of robustness to the major factors that can cause PCR failures: presence of inhibitors, degraded and low concentrated RNA.

Amplification has been performed using primer sets specifically designed for the amplification of the 3 PML-RARA translocations bcr1, bcr2, bcr3 and GUSβ as internal control. The amplification detection has been made possible by the use of either a DNA intercalating dye followed by annealing analysis or a labelled probe, a novel detection strategy developed in DiaSorin, which allows the real time fluorescence detection and the different transcripts discrimination.

A further improvement on the standard LAMP has been achieved with the introduction of a polymerase engineered in DiaSorin, able to couple the retrotranscriptional activity and the amplification step in one single tube. This allowed the one-step amplification starting directly from extracted RNA. This improved LAMP reaction has been called RT-Q-LAMP.

The final result, with the developed RT-Q-LAMP assays, can be obtained starting directly from extracted RNA in only 40 minutes from the beginning of the reaction, but, thanks to the introduction of real time detection, the signal of a positive sample can be visible right after 10-15 minutes.

1. PRIMER DESIGN AND SELECTION

Using two different softwares, *Primer Explorer* and *Visual OMP (*as described in Materials and Methods), several primer sets have been designed either for the amplification of the 3 PML-RARA translocations or for the internal control GUSβ.

Submitting the sequence of the PML-RARA translocation to *Primer Explorer*, the software returned a high number of sets for each target, but only a part of them followed in-between the breakpoint and could be candidate for the amplification of the translocation. Another application available in *Primer Explorer* allowed the selection of the loop primers for the favourite sets.

As next step in the primer selection process, in order to study the melting temperatures of the primers and the interactions between primers, the sets located in-between the translocations have been analysed with Visual OMP. The sets showing a dangerous presence of primer-primer interactions, potentially extending in the 3' end, have been excluded.

After this *in silico* selection, the remaining primer sets have been tested experimentally in the LAMP reaction. The reaction conditions have been set according to Notomi et al.[69] and the primer sets have been tested on the

specific plasmid, containing the target, down to low concentrations in order to test their sensitivity and on water samples to test the specificity. This step showed that around 20% of the primer sets did not produce any amplification within 40 minutes, therefore they have been excluded.

The primer sets have been ranked according to their speed and specificity. The majority of them showed to be fast enough, but a very low amount appeared to be also specific.

A summary showing the number of primer sets screened *in silico* and experimentally is shown in Figure 6.

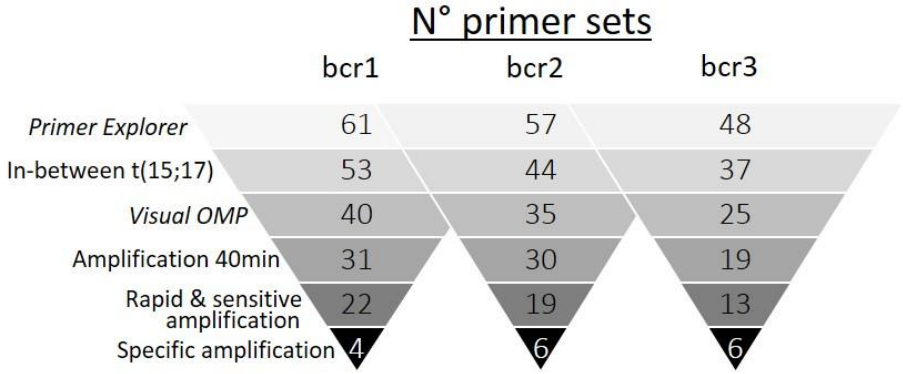

Figure 6. The pyramids show the number of primer sets successfully overcoming the different selection processes.

The primers for PML-RARA isoforms amplification, which have reached the end of the selection process, share a common localization on the constant portion of RARA exon 3, while the forward portions are located (Figure 7):

- for bcr1 on PML exon 6, with a portion of F1c in-between the breakpoint
- for bcr2 on PML exon 6, upstream to the variable part
- for bcr3 on PML exon 3, as close as possible to the breakpoint

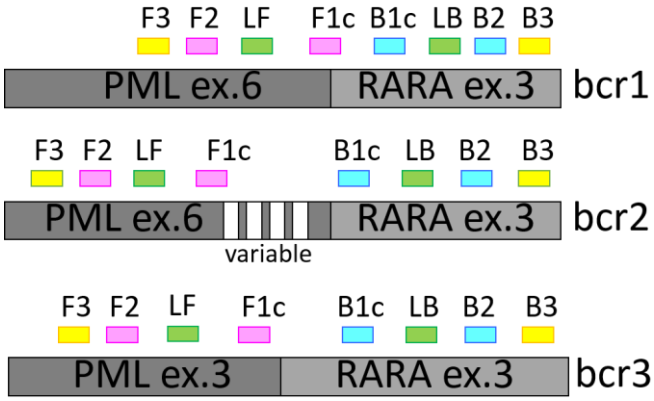

Figure 7. Position of the primer sets on the 3 PML-RARA isoforms.

For the amplification of the internal control GUSβ, the process of primer selection has followed the same steps, with the difference that, instead of being in-between a breakpoint, the primers have been selected for being in-between two exons. Different regions of GUSβ mRNA have been studied, but the final choice has been the sequence between exons 11 and 12 (Figure 8).

Figure 8. GUSβ primer design between exons 11 and 12.

Moreover, in the internal control primers selection, the speed of amplification was not a critical feature, because of the possible competition with the target amplification.

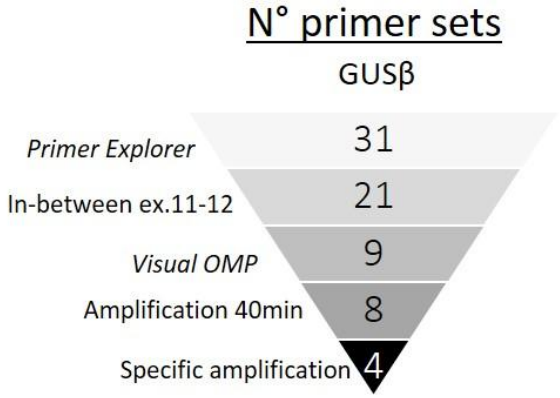

Figure 9. The pyramid shows the number of primer sets successfully overcoming the different selection processes for GUSβ primer design.

1.1 Evaluation of rapidity and sensitivity

As previously described, the selection of the best primers for each target has been based on the experimental performances. These performances have been evaluated by running LAMP reaction in the presence of an intercalating dye (YO-PRO-1® Iodide), followed by an annealing analysis.

The instrument Genie® II (Optigene) records the total fluorescence generated by YO-PRO1 binding to the dsDNA while the amplification occurs. The threshold time is calculated as the reaction time when the fluorescence overcomes the 50% threshold of the total fluorescence increase. Then, during the annealing step, the fluorescence is recorded as the temperature decreases from 98 to 85°C. The first derivative plot of the difference of

fluorescence in function of time will be a distinct peak. The annealing temperature of each product is defined as the temperature at which the corresponding peak occurs.

Even if at this stage the reaction is meant for the amplification of one single target (simplex reaction), the annealing analysis is critical for the definition of assay specificity, allowing the detection of primer dimers. Because of their small size, primer dimers usually anneal at lower temperatures than the desired product. Additionally, non-specific amplification on RNAs negative for the presence of the translocation PML-RARA may result in LAMP products that anneal at temperatures above or below that of the desired product.

The speed of amplification and the sensitivity of each primer set that passed the *in silico* selection has been evaluated performing a standard LAMP reaction on 4 different amounts of plasmid specific for each target: 10,000, 1,000, 100 and 10 copies per reaction.

The rapidity has been determined as the capability to complete the amplification of the 10 copies of plasmid within 40 minutes. Sensitivity has been defined as the percentage of replicates of 10 copies of plasmids detected within the end of the reaction (60 minutes) on the total number of replicates tested.

1.2 Evaluation of specificity

LAMP primers, especially FIPs and BIPs, are particularly long. Since in a LAMP reaction many primers coexist, it is possible that they form dimers,

which can give an amplification signal. In order to test the formation of primer dimers during the reaction, a No Target Controls (NTC), i.e. water, has been included in the screening of the primer sets.

The presence of non-specific amplification, due to alternative binding of primers to RNA sequences, has been evaluated testing RNA extracted from cells that do not carry PML-RARA mutations, such as HL-60. This screening was not possible for the internal control primer sets, being GUSβ a housekeeping gene.

Specificity has been defined as the percentage of samples (water or RNAs) not amplifying on the total number of replicates tested.

At the end of this selection flow an average of 10% of the initial number of primer sets has been found to be adequate for the next step, the development of the assays, by the multiplexing of the simplex reactions.

1.3 Selection of primers for multiplex reactions

The primers that reached the bottom of the pyramid (Figures 6 and 9) have been crossed in order to obtain a triplex reaction (bcr1, bcr3 and GUSβ) and a duplex reaction (bcr2 and GUSβ). This created a high number of combinations that needed to be screened again, according to approximately the same criteria described previously.

The *in silico* selection has been made thanks to *Visual OMP* software, evaluating the formation of extending structures between primers. This has allowed a first strong decrease of the possible combinations.

Following the *in silico* selection, primer sets have been chosen according to their rapidity, sensitivity and specificity, evaluated experimentally.

In this phase the annealing analysis has allowed the correct discrimination of the target amplified. In fact each PML-RARA transcript, thanks to the different nucleotidic sequence, showed to have a peculiar annealing temperature as follows: bcr1 (91°C), bcr2 (90°C) and bcr3 (93°C). Importantly these annealing temperatures are also different from the GUSβ one, since its target sequences have been specifically selected in an AT reach region, in order to have a low annealing temperature (88°C).

At the end of the selection procedure, 7 and 6 combinations of primer sets have been selected respectively for the triplex and duplex assays. Then, in order to reduce the cost in the development phases, the same primer set for the internal control of the two assays has been selected (Figure 10).

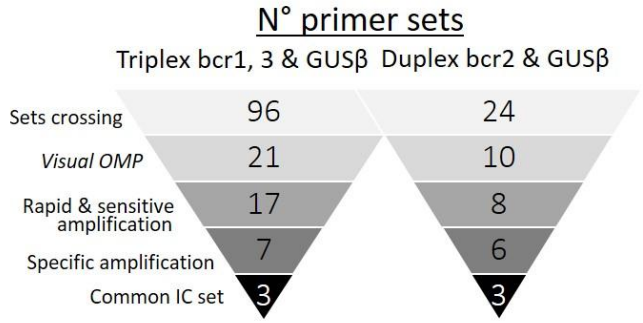

Figure 10. The pyramids show the number of primer sets successfully overcoming the different selection processes for the two assays.

1.4 Fluorescent probes

In order to take advantage of the great speed of a LAMP reaction, the multiplex reactions have been developed with the introduction of a

fluorescent probe. This improvement allowed the real time detection and discrimination of the amplification of the 3 transcripts and of the internal control, without waiting for the annealing analysis.

The fluorescent probe has replaced one of the loop primers. This also allowed the further selection between the 3 combinations of primer sets, choosing the one in which the exclusion of one loop primer had the minor impact on sensitivity.

The labelled probes have been substituted to LF for bcr1 and bcr3 and to LB for bcr2 and GUSβ. In the triplex reaction, bcr1 has been labelled with CHROMIS 530, bcr3 with CHROMIS 570 and GUSβ with CHROMIS 500. In the duplex reaction, bcr2 has been labelled with CHROMIS 500 and GUSβ with CHROMIS 530.

2. RT-Q-LAMP MULTIPLEX REACTIONS OPTIMIZATION

Once the primer sets have been selected, a deep optimization work needed to be started in order to obtain the best results in terms of sensitivity and specificity.

For the sensitivity evaluation the only cell line available is NB-4, carrying bcr1 mutation. Therefore, for bcr2 and bcr3 the sensitivity has been evaluated on plasmids diluted into RNA from HL-60 cell line, which is negative for PML-RARA translocation.

Analytical specificity has been evaluated during the optimization on water and RNAs negative for PML-RARA.

In LAMP reaction many primers and reagents coexist to allow the occurrence of a very complex reaction. During the optimization process each reagent has been modulated until the optimal formulation has been obtained.

2.1 Primer concentration

The standard primer concentrations of LAMP are indicated in the Materials & Methods and I will refer here at those concentrations as "1X".

In order to evaluate the performances of the primer sets the concentrations have been reduced and increased, obtaining a speed up of the reaction at higher concentrations, reaching a plateau after which a saturation of the reaction has probably occurred (Figures 11 and 12).

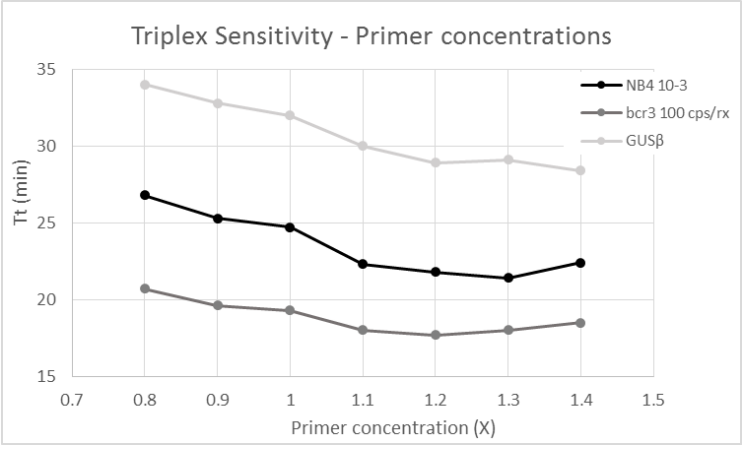

Figure 11. Impact of primers concentration on sensitivity in the triplex bcr1, 3 assay. The speed of the reaction decreases until a plateau is reached, after which the reaction speed can sometimes be negatively affected by primer overloading.

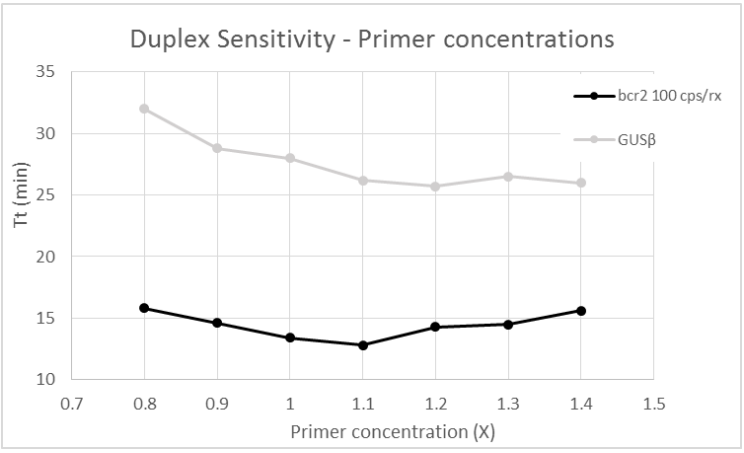

Figure 12. Impact of primers concentration on sensitivity in the duplex bcr2 assay. The speed of the reaction decreases until a plateau is reached, after which the reaction speed can be negatively affected by primer overloading.

The variation of primers concentration had a significant impact on specificity, especially on primer dimers, but it was not strictly correlated to the higher concentration of primers in both the assays (Figures 13 and 14).

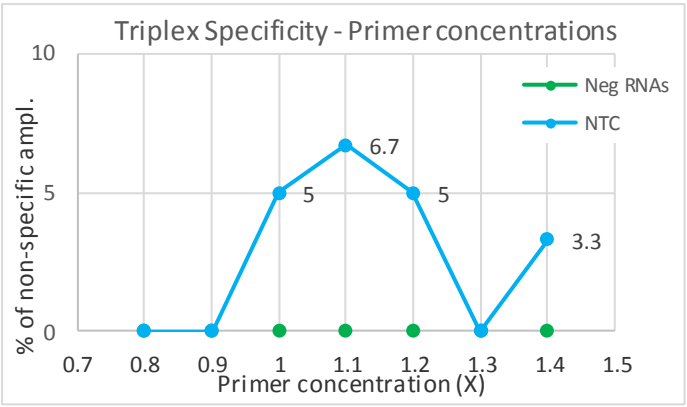

Figure 13. Impact of primers concentration on specificity in the triplex bcr1, 3 assay. The amount of non-specific signals is not strictly correlated to the increased primers concentrations.

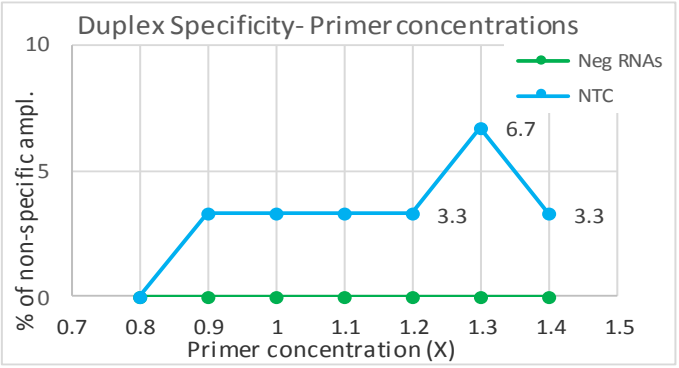

Figure 14. Impact of primers concentration on specificity in the duplex bcr2 assay. The amount of non-specific signals is quite constant for all the tested concentrations, not strictly correlated to the increased primers concentrations.

2.2 dNTPs concentration

During the optimization, a gradient of dNTPs concentration has been tested. In the standard LAMP conditions the dNTPs concentration is 1.4 mM. Different concentrations around this value have been tested, finding a clear speed up trend at the increasing of dNTPs concentration (Figures 15 and 16). The specificity has not been influenced by the dNTPs amount, neither on NTCs or negative RNAs.

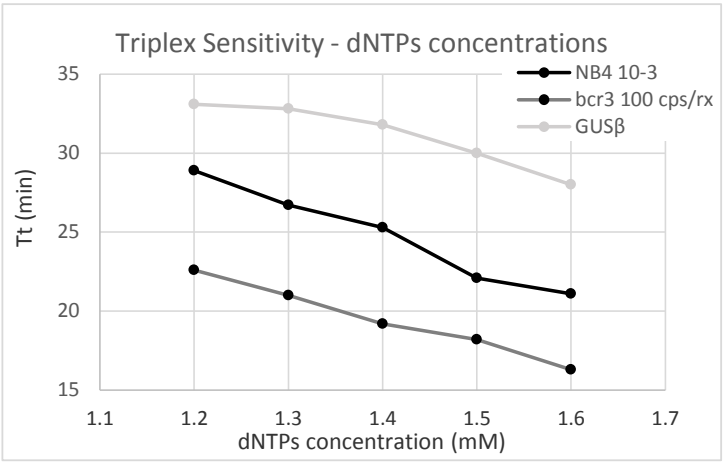

Figure 15. Impact of dNTPs concentration on sensitivity in the triplex bcr1, 3 assay. The reaction speeds up at the increasing of dNTPs concentration.

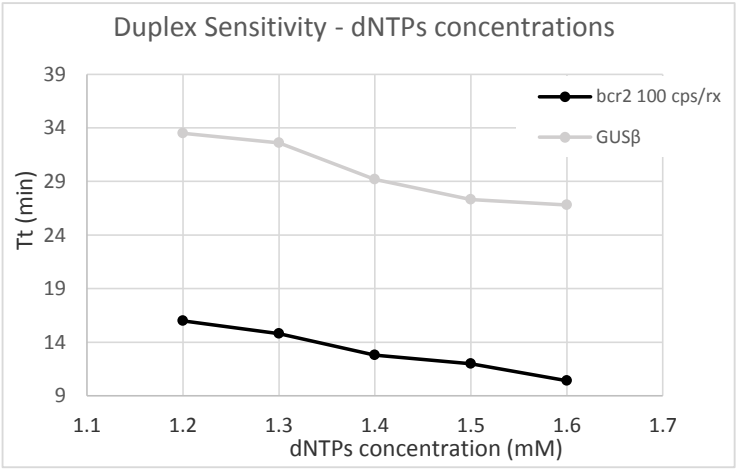

Figure 16. Impact of dNTPs concentration on sensitivity in the duplex bcr2 assay. The reaction speeds up increasing dNTPs concentration.

2.3 Manganese concentration

During the optimization, a gradient of $MnCl_2$ concentration has been tested. In the standard LAMP conditions the $MnCl_2$ concentration is 4 mM. Different concentrations around this value have been tested, finding a slight impact on reaction speed, with a minor acceleration at lower concentrations only on the triplex assay (Figure 17 and 18). Otherwise, specificity has been significantly affected by the increasing of $MnCl_2$ concentration, which caused the appearance of non-specific signals (Figures 19 and 20).

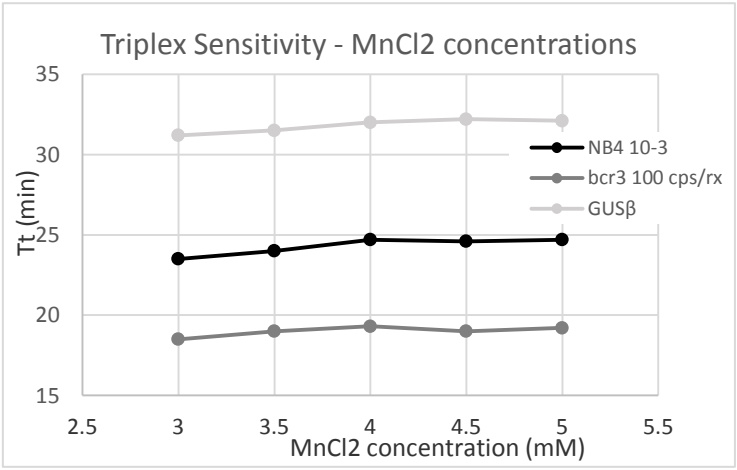

Figure 17. Impact of $MnCl_2$ concentration on sensitivity in the triplex bcr1, 3 assay. The reaction has a slight speed up at lower concentrations.

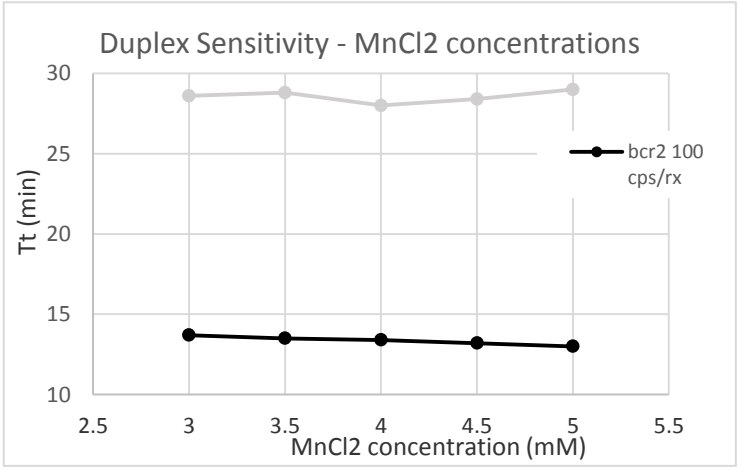

Figure 18. Impact of $MnCl_2$ concentration on sensitivity in the duplex bcr2 assay. No significant influence of $MnCl_2$ variations is detected in the reaction.

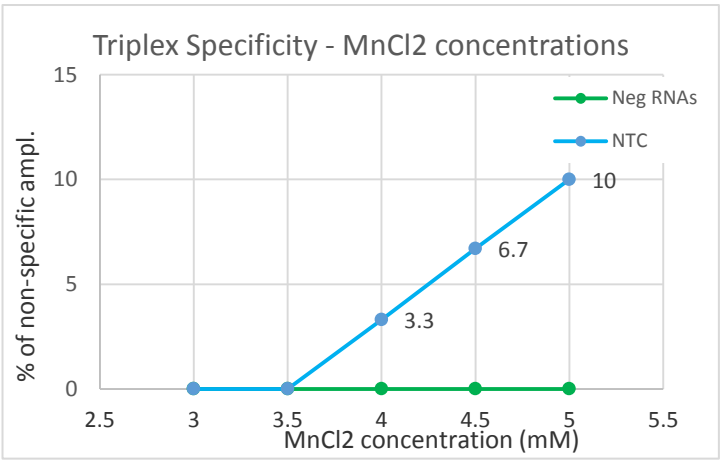

Figure 19. Impact of $MnCl_2$ concentration on specificity in the triplex bcr1, 3 assay. A constant increase in primer dimers is detectable at higher concentrations of $MnCl_2$.

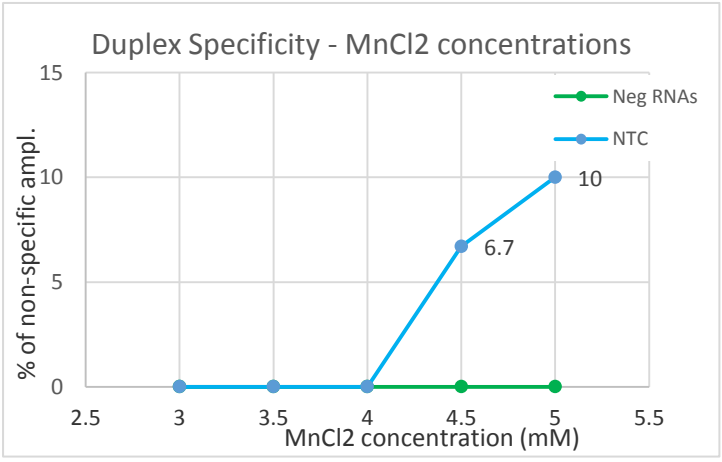

Figure 20. Impact of $MnCl_2$ concentration on specificity in the duplex bcr2 assay. A constant increase in primer dimers is detectable at higher concentrations of $MnCl_2$.

2.4 Enzyme concentration

During the optimization, different amounts of enzymes have been tested. In the standard LAMP conditions the concentration of the enzyme is 8 U/rx. Different concentrations around this value have been tested, finding an increase of reaction speed at higher concentrations for both the assays (Figures 21 and 22). Unfortunately, in parallel to the speed up, an increase of primer dimers and non-specific signals was detected (Figures 23 and 24).

In the entire optimization process, the concentration of enzyme has been the parameter with the higher impact on the reaction performances.

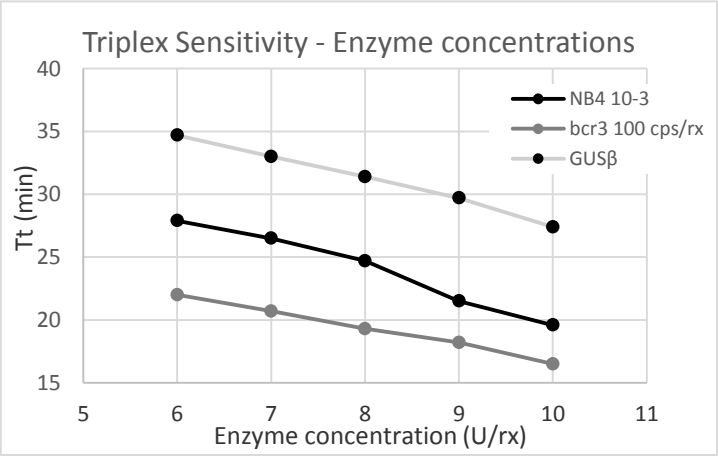

Figure 21. Impact of enzyme concentration on sensitivity in the triplex bcr1,3 assay. A significant speed up in all the targets has been obtained increasing the amount of enzyme in the reaction.

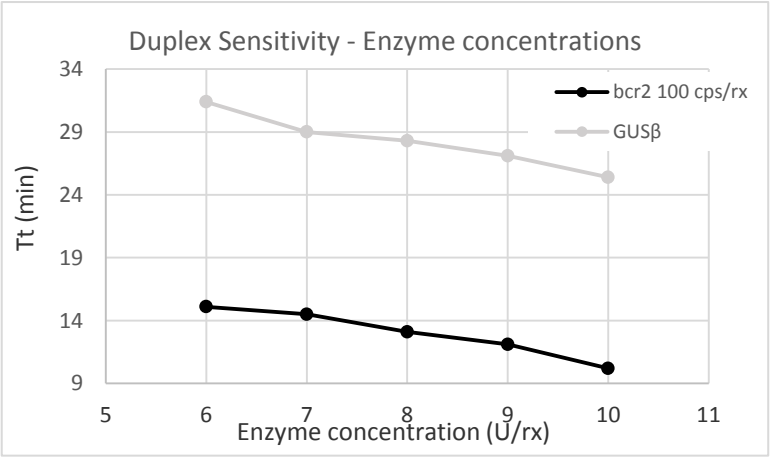

Figure 22. Impact of enzyme concentration on sensitivity in the duplex bcr2 assay. A significant speed up in both the targets has been obtained increasing the amount of enzyme in the reaction.

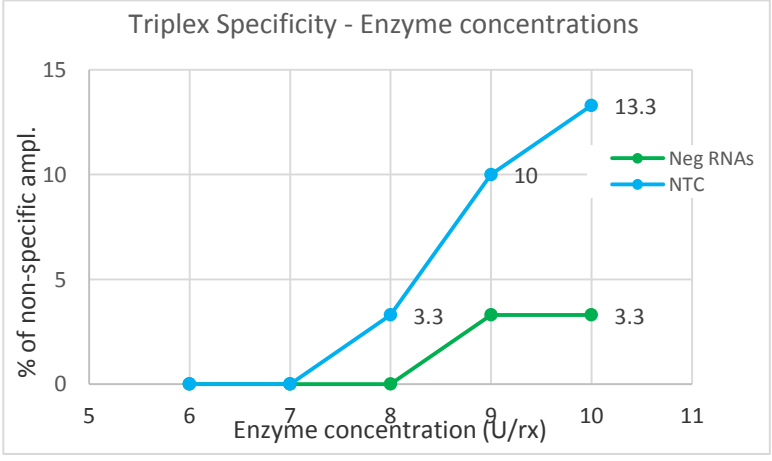

Figure 23. Impact of enzyme concentration on specificity in the triplex bcr1, 3 assay. A significant increase in non-specific signals and primer dimers is detected at the increasing of enzyme concentrations.

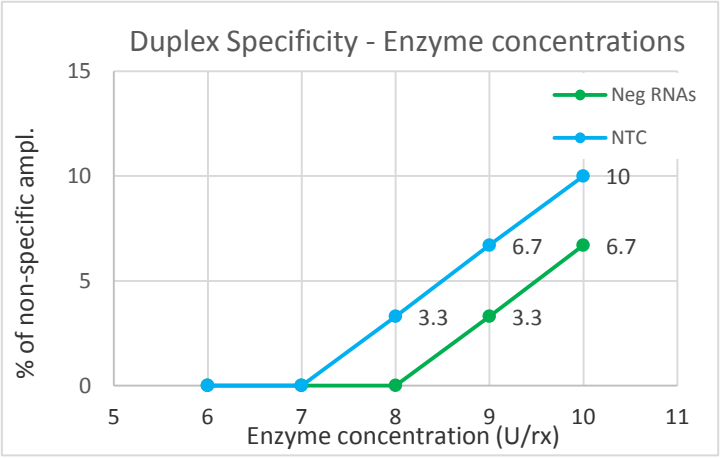

Figure 24. Impact of enzyme concentration on specificity in the duplex bcr2 assay. A significant increase in non-specific signals and primer dimers is detected at the increasing of enzyme concentrations.

2.5 Buffer pH

During the development of the assays it has been discovered that also the pH of the reaction buffer has an impact on the functionality of the enzyme, therefore a gradient of pH has been tested during the optimization phase.

The impact of pH has been clearly detectable only on the amplification of the internal control GUSβ (Figures 25 and 26) that is delayed by the acidification of the reaction buffer. This is probably due to a sequence-specific impact of the pH on the primers in the reaction mix. Despite the speed up of the internal control at more basic pH, no issues of reduction of sensitivity have been observed for the bcr1, bcr3 or bcr2 positive samples.

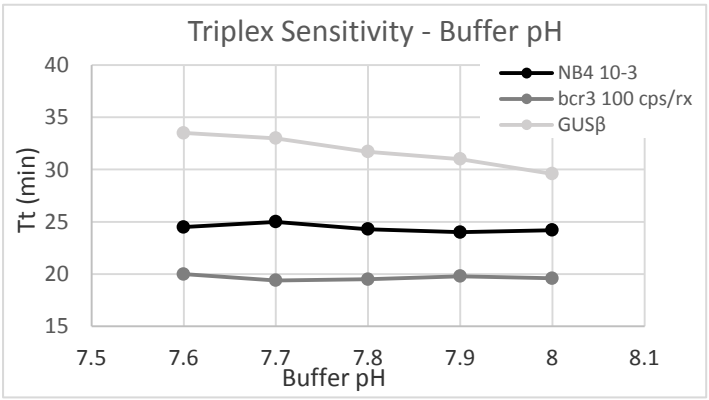

Figure 25. Impact of buffer pH on sensitivity in the triplex bcr1,3 assay. A significant speed up is detected only in the internal control amplification. No impact is clearly detectable on bcr1 or bcr3 amplification.

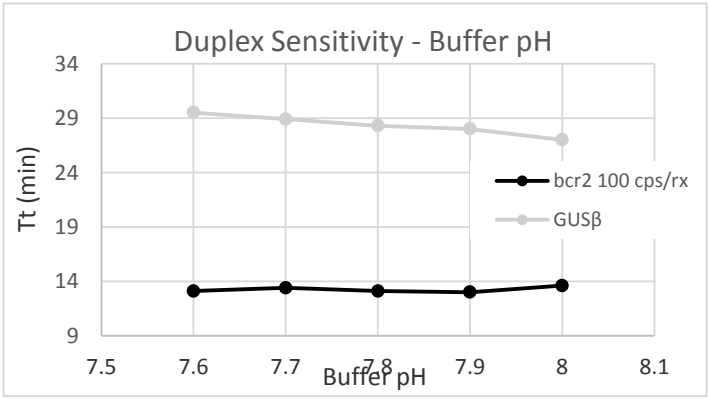

Figure 26. Impact of buffer pH on sensitivity in the duplex bcr2 assay. A significant speed up is detected only in the internal control amplification. No impact is clearly detectable on bcr2 amplification.

2.6 Reaction temperature

Primers have been designed with optimal Tm for a reaction running at 65°C. In order to study the impact of temperature on the functionality of primers and enzyme, different reaction temperatures have been tested, in a range around 65°C. This study demonstrated that at lower temperatures the primers could work properly, but, as the temperature increased, their efficiency was partially affected. This has been observed in particular in the internal control amplification (Figure 27), whose primers have a lower Tm, being designed in an AT rich region.

At higher temperatures there is a positive effect on specificity, with the prevention of formation of primer dimers that at lower temperatures can sometimes be detected (Figure 28).

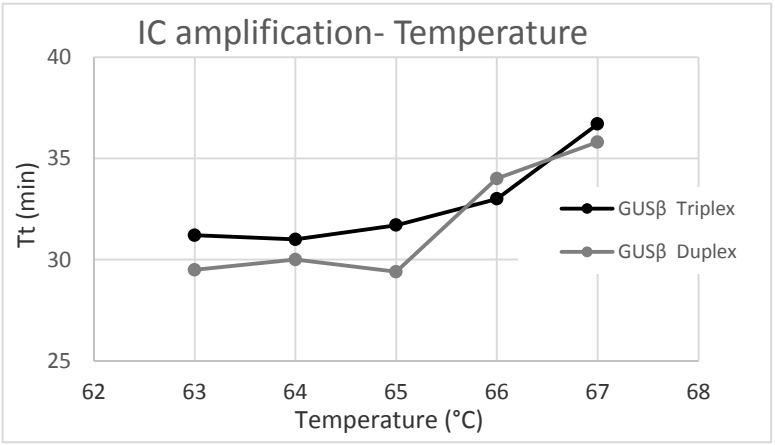

Figure 27. Impact of the reaction temperature on the amplification of the internal control in the two assays. GUSβ is significantly delayed by the increasing of the temperature.

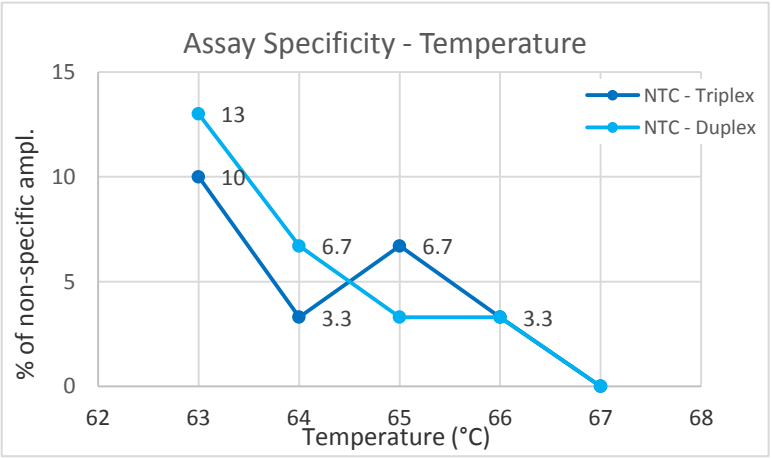

Figure 28. Impact of the reaction temperature on the specificity of the two assays. The increase of temperature avoids the formation and amplification of primer dimers that, at 67° are completely absent.

3. RT-Q-LAMP MULTIPLEX REACTIONS FREEZE-DRYING

The optimization process has involved hundreds of different combinations of all the factors described above until the best formulations for the two assays were found. Due to the simultaneous occurrence of two or three independent reactions, the reactions for the amplification of the targets and of the internal control had to be well balanced to ensure the correct use of common reagents, avoiding the taking over of one system on the others.

These final formulations have then been freeze-dried in order to have several advantages such as the easy set-up, the higher reproducibility between experiments, the more standardized production process and the longer shelf life at room temperature.

The freeze-drying process starts with the freezing of the reaction mix and the subsequent sublimation of the water contained in the mix. This process can alter the characteristics of the reaction mix leading to problems in sensitivity or specificity.

For both assays, no changes in reaction sensitivity or speed have been detected, but for the duplex assay a major issue of specificity has been detected. In the freeze-dried format almost 100% of negative RNAs and water samples has given a non-specific signal in the 500 nm channel. In the liquid format, specificity had been previously tested on 108 replicates, with only a small percentage of non-specific signals detected in both channels (Figure 29).

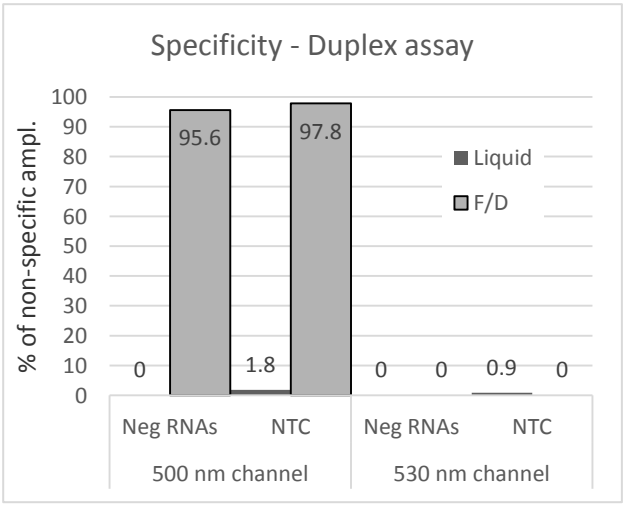

Figure 29. Impact of the freeze-drying process on duplex assay specificity. The presence of primer dimers and non-specific signals severely increases when the reaction mix undergoes the freeze-drying (F/D) step.

In order to understand the cause of this very high rate of non-specific signals, an experimental analysis has been performed, excluding each

primer from the freeze-dried mix and testing the specificity of each combination on 20 replicates of NTCs. This has allowed the identification of bcr2 LF as the responsible for all non-specific amplifications (Figure 30). This single primer has been therefore re-designed and tested in the freeze-dried formulation, until the optimal sequence has been identified.

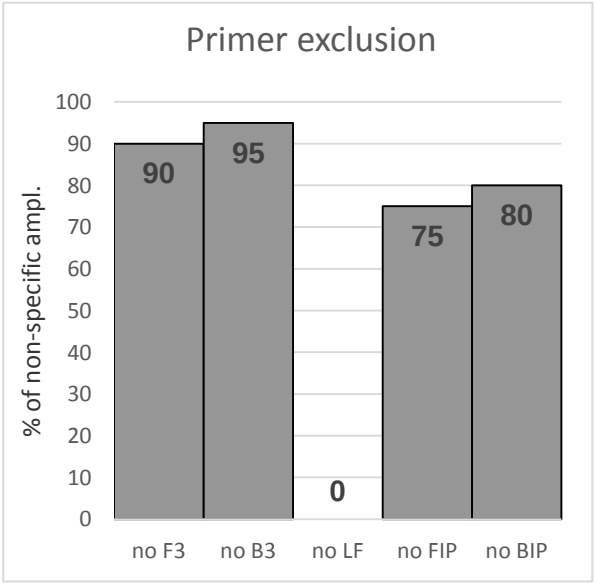

Figure 30. Identification of the primer responsible for non-specific amplifications. The exclusion of LF primer from the freeze-dried mix restored the condition of specificity of the liquid mix.

4. RT-Q-LAMP MULTIPLEX REACTIONS PERFORMANCES

The following results have been obtained with the final freeze-dried formulations of the triplex bcr1, bcr3 & GUSβ assay and duplex bcr2 & GUSβ assay.

4.1 Analytical sensitivity

Analytical sensitivity is determined by the Limit of Detection (LoD) value, which represents the lower mutation dose that can be detected in at least 95% of replicates. The performances of the triplex assay bcr1/bcr3/GUSβ have been evaluated using 500 ng/reaction of total RNA.

The sensitivity on bcr1 transcript has been assessed using RNA extracted from NB-4 cell line, serially diluted into RNA extracted from HL-60 cell line, which does not carry any PML-RARA translocation. The signal for bcr1 has been detected in 530 nm channel on Liaison IAM instrument down to 1:10,000 dilution of NB-4 RNA into HL-60 (NB4 10^{-4}) (Table 6). The LoD for bcr1 transcript is NB4 10^{-3} dilution, detected in 100% of a high number or replicates (Table 6).

The time to result is 40 minutes, but the signal of undiluted NB-4 RNA could be detected right after 10 minutes from the start of the reaction, with a mean threshold time of 13.57 minutes (Table 6 and Figure 31).

Sample	Replicates detected	Sensitivity	AVG Tt (min)	STD DEV (min)
NB-4	22/22	100%	13.57	0.61
NB-4 10^{-1}	28/28	100%	15.78	0.89
NB-4 10^{-2}	102/102	100%	17.27	1.25
NB-4 10^{-3}	116/116	100%	21.50	3.65
NB-4 10^{-4}	37/68	54%	27.65	5.66

Table 6. Sensitivity of PML-RARA bcr1/3 assay on NB-4 cell line

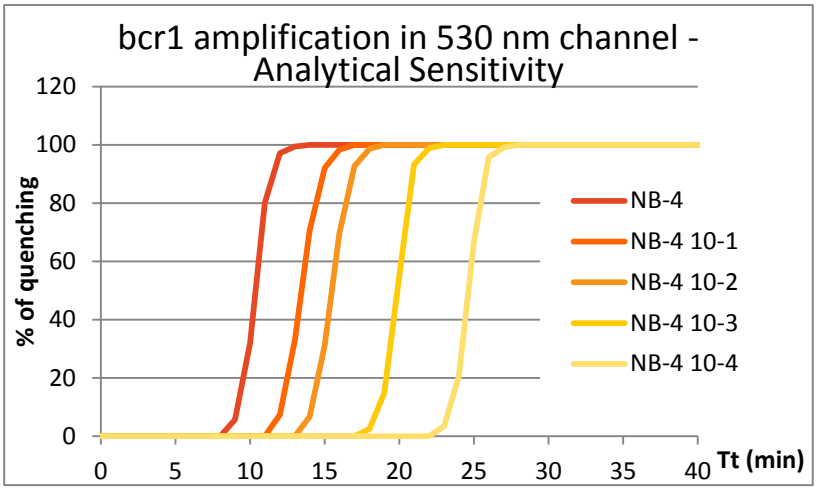

Figure 31. Example of amplification of bcr1 positive samples (NB-4) at different dilutions. The mutation load is inversely proportional to the threshold time.

NB-4 and HL-60 RNAs have been extracted with three different extraction methods: Qiagen columns, phenol-chloroform and Promega Maxwell®. The amplification results in terms of sensitivity and threshold times have not significantly been affected by the different extraction methods, as shown in Table 7.

	Qiagen extraction			Phenol-chloroform extraction			Maxwell®Promega extraction		
Sample	**Reps detected**	**AVG Tt (min)**	**STD DEV (min)**	**Reps detected**	**AVG Tt (min)**	**STD DEV (min)**	**Reps detected**	**AVG Tt (min)**	**STD DEV (min)**
NB-4	22/22	13.57	0.61	7/7	12.98	0.32	5/5	13.44	0.20
NB-4 10^{-1}	28/28	15.78	0.89	15/15	16.28	0.41	5/5	16.02	0.42
NB-4 10^{-2}	102/102	17.27	1.25	40/40	17.34	0.58	10/10	18.03	0.99
NB-4 10^{-3}	116/116	21.50	3.65	40/40	20.62	3.09	12/12	22.37	1.84

Table 7. Sensitivity of PML-RARA bcr1/3 assay on NB-4 cell line with different extraction methods.

On bcr3 transcript the analytical sensitivity could not be evaluated on RNA, but a model, as close as possible to a cell line, has been developed by spiking the bcr3 plasmid into HL-60 RNA. The plasmid has been serially diluted into RNA down to 5 copies per reaction. The signal for bcr3 has been detected in 570 nm channel on Liaison IAM instrument down to 5 copies per reaction (Table 8). The LoD for bcr3 transcript is 30 copies per reaction diluted into HL-60 RNA, detected in 99.1% of the replicates.

The time to result is 40 minutes, but the signal of 10,000 copies of bcr3 plasmid into HL-60 RNA could be detected right after 15 minutes from the start of the reaction, with a mean threshold time of 16.04 minutes (Table 8 and Figure 32).

bcr3 plasmid cps/rx in HL-60 RNA	Replicates detected	Sensitivity	AVG Tt (min)	STD DEV (min)
10^4	20/20	100%	16.04	0.63
10^3	20/20	100%	17.55	0.87
10^2	22/22	100%	19.33	1.16
30	106/107	99.1%	21.67	2.69
10	42/48	87.5%	23.36	3.51
5	5/19	26.3 %	26.80	5.82

Table 8. Sensitivity of PML-RARA bcr1/3 assay on bcr3 plasmid spiked into HL-60 RNA.

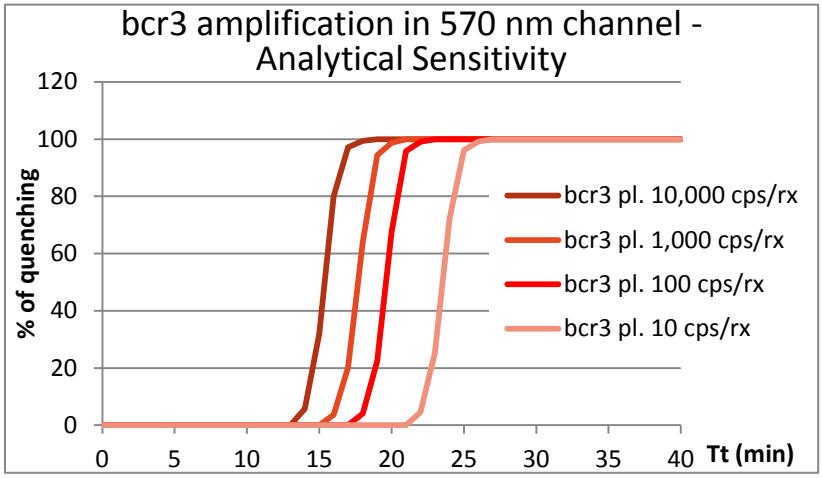

Figure 32. Example of amplification of bcr3 positive samples (bcr3 plasmid diluted in HL-60 RNA) at different copy numbers. The mutation load is inversely proportional to the threshold time.

As for bcr3, bcr2 plasmid has been spiked into HL-60 RNA for the assessment of sensitivity. In the bcr2/GUSβ duplex reaction, bcr2 signal could be detected in 500 nm channel on the Liaison IAM instrument down to 5 copies per reaction. The LoD for bcr2 transcript is 10 copies per

reaction diluted into HL-60 RNA, detected in 100% of a high number of replicates (Table 9).

The time to result is 40 minutes, but the signal of 10,000 copies of bcr2 plasmid into HL-60 RNA could be detected right after 9 minutes from the start of the reaction, with a mean threshold time of 11.54 minutes (Table 9 and Figure 33).

bcr2 plasmid cps/rx in HL-60 RNA	Replicates detected	Sensitivity	AVG Tt (min)	STD DEV (min)
10^4	18/18	100%	11.54	0.65
10^3	15/15	100%	12.64	0.48
10^2	22/22	100%	14.53	1.34
10	112/112	100%	17.22	3.33
5	50/53	94.3%	22.80	5.62

Table 9. Sensitivity of PML-RARA bcr2 assay on bcr2 plasmid spiked into HL-60 RNA.

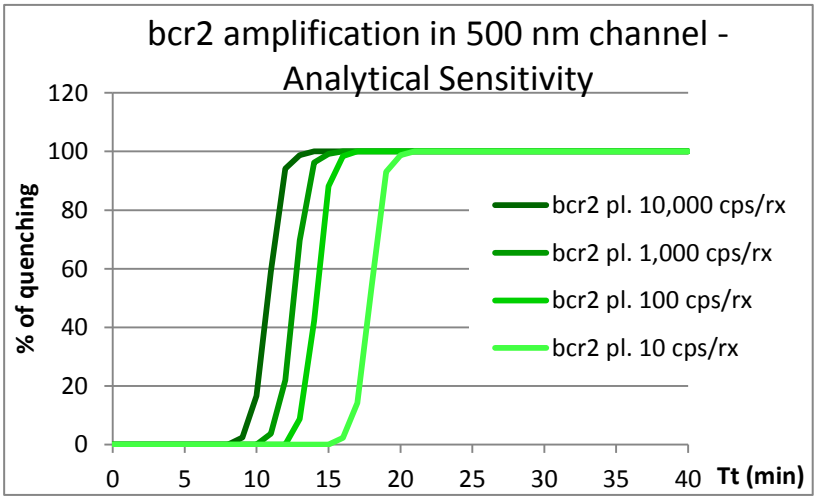

Figure 33. Example of amplification of bcr2 positive samples (bcr2 plasmid diluted in HL-60 RNA) at different copy numbers. The mutation load is inversely proportional to the threshold time.

4.2 Analytical specificity on negative RNAs

Specificity of the assays has been assessed using 500 ng per reaction of RNA extracted from 8 different cell lines, negative for PML-RARA translocations (HL-60, REH, 697, TOM-1, K-562, KASUMI-1, MV4-11 and RS4;11).

Only the GUSβ signal, in 500 nm channel for the triplex and in 530 nm channel for the duplex, has been detected on Liaison IAM instrument, on a high number of replicates, with an average threshold time of 25.61 minutes for the triplex assay and 29.85 minutes for the duplex assay (Table 10).

PML-RARA bcr1/3 assay			
Negative cell line	**# of replicates**	**AVG Tt (min)**	**STD DEV (min)**
HL-60	104	25.13	0.12
REH	20	25.83	0.22
TOM-1	20	25.70	0.14
K-562	20	24.88	0.10
KASUMI-1	20	26.10	0.12
MV4-11	20	24.78	0.13
RS4;11	20	26.17	0.17
TOT	**224**		

PML-RARA bcr2 assay			
Negative cell line	**# of replicates**	**AVG Tt (min)**	**STD DEV (min)**
HL-60	230	28.77	0.28
REH	20	31.11	0.44
TOM-1	20	29.81	0.37
K-562	20	30.31	0.87
KASUMI-1	20	29.08	0.51
MV4-11	20	30.30	0.86
RS4;11	20	29.18	0.87
TOT	**350**		

Table 10. Specificity of PML-RARA assays on RNA extracted from negative cell lines.

As for sensitivity, also specificity has been evaluated on RNA extracted with three extraction methods: Qiagen columns, phenol-chloroform and Maxwell® Promega. These three methods had no significant impact on specificity or on GUSβ threshold times (Table 11, 12). Only few cell lines

extracted with phenol-chloroform were available, and only HL-60 extracted with Maxwell® Promega.

	PML-RARA bcr1/3 assay					
	Phenol-chloroform extraction			Maxwell®Promega extraction		
Sample	Replicatesd etected	AVG Tt (min)	STD DEV (min)	Replicates detected	AVG Tt (min)	STD DEV (min)
HL-60	10/10	23.92	0.30	30/30	23.94	0.34
TOM-1	10/10	26.11	0.18			
K-562	10/10	25.57	0.22			

Table 11. Analytical specificity of PML-RARA bcr1/3 assay on cell lines extracted with phenol-chloroform and Maxwell® Promega methods.

	PML-RARA bcr2 assay					
	Phenol-chloroform extraction			Maxwell®Promega extraction		
Sample	Replicatesd etected	AVG Tt (min)	STD DEV (min)	Replicates detected	AVG Tt (min)	STD DEV (min)
HL-60	10/10	28.99	0.65	30/30	29.32	0.27
TOM-1	10/10	30.18	1.06			
K-562	10/10	28.74	0.66			

Table 12. Analytical specificity of PML-RARA bcr2 assay on cell lines extracted with phenol-chloroform and Maxwell® Promega methods.

4.3 Analytical specificity on NTCs

The combination of a higher number of primers in the multiplex reactions made necessary the evaluation of specificity also on No Target Controls (i.e. water).

Both the assays in the final formulation demonstrated to be highly specific, showing any non-specific amplification in any of the channels (Table 13).

Amplification channel	PML-RARA bcr1/3 assay	PML-RARA bcr2 assay
500 nm	0/409	0/549
530 nm	0/409	0/549
570 nm	0/409	-

Table 13. Analytical specificity of PML-RARA assays on No Target Controls.

5. COMPARISON BETWEEN RT-Q-LAMP AND RT-PCR ON CLINICAL SAMPLES

After the first phase, in which analytical specificity and sensitivity has been evaluated on plasmids and cell lines, the assays have been tested on real clinical samples, previously tested with the reference method RT-PCR.

The samples have been collected and tested for RT-PCR in two of the main Italian centres for the diagnosis and treatment of APL, Azienda Ospedaliera Papa Giovanni XXIII, in Bergamo, and Policlinico Tor Vergata, in Rome.

5.1 Relative sensitivity on positive clinical samples

91 RNAs of positive clinical samples extracted from cells isolated from bone marrow or peripheral blood, with both Qiagen columns and phenol-chloroform method, have been examined. All these samples have shown to be positive for PML-RARA translocations with both RT-Q-LAMP and RT-PCR.

Due to the high efficiency of LAMP primers bcr2 samples are detected also with in bcr3 assay, which can amplify a dumbbell close to 500 bp. For the same reason, bcr2 assay can easily amplify bcr1 positive samples, as the 2 transcripts differ for less than 200 bp. Due to this characteristics the results of the two assays need to be combined in order to give the correct discrimination of the isoforms (Table 14).

bcr1	bcr3	bcr2	Final result
+	-	+/-	bcr1
-	+/-	+	bcr2
-	+	-	bcr3

Table 14. Table for results interpretation.

RT-Q-LAMP demonstrated to be more accurate on the discrimination of the transcripts. On 11 bcr2 positive samples assigned with RT-PCR, RT-Q-LAMP has detected the presence of bcr2 transcript in only 3 of them, while the others were bcr1 positive samples, as then confirmed by sequencing analysis.

Moreover, two discordant results have been initially identified:

- **ID5322**, has been amplified by qualitative RT-PCR as bcr1/2, the following RQ-PCR monitoring showed a higher copy number for bcr2 than bcr1, therefore the sample has been classified as bcr2

positive. This sample has been detected by RT-Q-LAMP as bcr1 positive.

- **ID2514**, has been classified by RT-PCR as bcr3 positive and detected by RT-Q-LAMP as bcr1 positive

These discordances had to be resolved by a third method, sequencing, which showed:

1. in sample **ID5322** a deletion of 24 bp plus 3 point mutations. Two of them are in the 3' end of ENF903 primer used for bcr1 RQ-PCR and are the possible cause of the delayed amplification. LAMP primers are not interested by these mutations, for this reason the sample has been correctly amplified as bcr1 positive;
2. in sample **ID2541** the deletion of PML exon 5. This sample did not amplify with bcr1/2 qualitative primers because they anneal on PML exon 5.

Both these samples were unusual and rare variants, but have been correctly and promptly amplified with PML-RARA LAMP assays.

All the positive samples have been amplified before 20 minutes, with an average of 15.25 minutes for bcr1, 17.84 minutes for bcr2 and 18.64 minutes for bcr3 (Table 15). The higher standard deviation for bcr2 is due to the difference in length of the transcript in each patient.

In Table 15 are reported also the average threshold times of bcr1 samples amplified with PML-RARA bcr2 assay and the bcr2 samples detected with PML-RARA bcr1/3 assay in bcr3 detection channel.

		PML-RARA bcr1/3 assay		PML-RARA bcr2 assay	
Translocation	**# of samples**	**AVG Tt (min)**	**SD**	**AVG Tt (min)**	**SD**
bcr1	43	15.25	0.51	18.05	2.66
bcr2	11	28.53	3.25	17.84	2.51
bcr3	37	18.63	0.52	-	-

Table 15. Relative sensitivity on bcr1, bcr2 and bcr3 positive clinical samples of PML-RARA assays

The sensitivity of the two assays has been tested also on serial dilutions of clinical samples. One sample for each transcript has been serially diluted down to 1:1,000 in HL-60 RNA and each dilution tested in 15 replicates. The triplex on bcr1 and bcr3 reached the LoD on 10^{-3} dilutions while the duplex on bcr2 reached the LoD on 10^{-2} dilution (Table 16).

Sample	Replicates detected	AVG Tt (min)	SD
BM1685/12 10^{-3} (bcr1+)	15/15	19.77	1.23
BM1176/12 10^{-3} (bcr3+)	15/15	23.75	3.95
BM512/12 10^{-3} (bcr2+)	11/15	28.41	4.52
BM512/12 10^{-2} (bcr2+)	15/15	24.16	1.47

Table 16. Relative sensitivity on bcr1, bcr2 and bcr3 positive samples diluted into HL-60 RNA.

5.2 Relative specificity on negative clinical samples

In order to test the specificity of our assays on negative clinical samples, we examined 105 negative RNA samples from healthy donors (n=54) and patients affected by other hematological pathologies (n=51).

These samples have been tested with freeze-dried reaction mix with both the assays, confirming the negative result obtained with the reference method RT-PCR.

For all the samples, the internal control GUSβ correctly amplified, with an average of 26.45 minutes in the triplex assay and 28.71 minutes in the duplex assay.

RNA has been extracted with three different methods, Qiagen columns, phenol-chloroform and Maxwell® Promega. No significant difference has been detected between the amplification of the three groups of samples (Table 17).

	PML-RARα bcr1/3 assay			PML-RARα bcr2 assay		
Extraction method	**Reps detected**	**AVG Tt (min)**	**STD DEV (min)**	**Reps detected**	**AVG Tt (min)**	**STD DEV (min)**
Qiagen	52/52	25.22	0.57	52/52	31.01	1.19
Phenol-chloroform	42/42	25.63	0.30	42/42	28.82	0.61
Maxwell® Promega	10/10			10/10		

Table 17. Relative specificity on negative clinical samples.

6. ROBUSTNESS OF RT-Q-LAMP ASSAYS

Thanks to its efficiency, RT-Q-LAMP demonstrated to be more robust than PCR in different conditions. The performances of the PML-RARA assays have been challenged in the presence of inhibitors, degraded RNA samples and very low concentrated RNAs.

6.1 Robustness to inhibitors

One of the major causes of inhibition for PCR is chemical contamination by ethanol or phenol that remain present in the final extracted RNA. These contaminants can be detected by reading the sample with a spectrophotometer at 230 nm. The ratio between the absorbance values at 260 nm and 230 nm gives an indication of the contamination level. An RNA is considered optimal when this ratio is between 1.8 and 2.0.

In order to test robustness of LAMP technology to such contaminants, 40 RNAs have been tested, NB4 as positive samples and negative cell lines with a very low 260/230 (0.29-0.99).

All the samples have been amplified without any significant delay in threshold times for both bcr1 and internal control (Table 18), showing robustness to chemical contamination.

Sample	Replicates detected	AVG Tt (min)	SD
NB-4	12/12	14.22	0.21
HL-60	8/8	24.75	0.17
RS4;11	11/11	26.56	0.54
REH	9/9	25.46	0.47

Table 18. Amplification of positive (NB-4) and negative (HL-60, RS4;11 and REH) samples with low 260/230, indicating chemical contamination.

Moreover, 2 clinical samples which could not be amplified by RT-PCR because of a possible inhibition have been tested by RT-Q-LAMP. RT-PCR had to be repeated because the amplification of the external control ABL was not strong enough to allow the validation of the result, despite the correct amplification of bcr1 and bcr3 positive controls (Figure 34). The RT-PCR has been repeated several times, confirming this result. As a consequence the sample had to be re-collected and extracted.

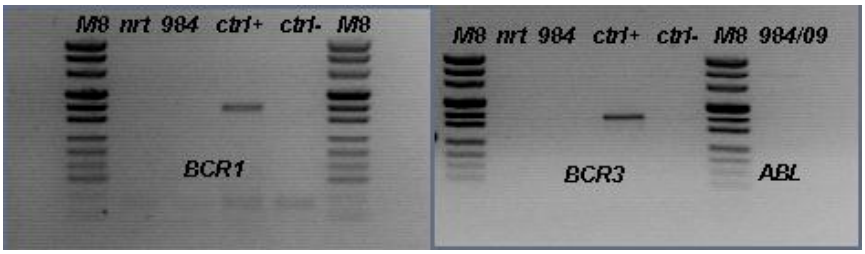

Figure 34. PCR result for patient BM984/14.

With RT-Q-LAMP the samples have been correctly amplified (Table 19) and the threshold times not affected by any inhibitory effect.

Sample	Transcript type	Tt (min)
BM984/09	bcr1	13.85
BM62/15	negative	24.40

Table 19. RT-Q-LAMP performances on samples in presence of inhibitors.

6.2 Robustness to degraded samples

In order to test the performances of the PML-RARA RT-Q-LAMP assays on degraded samples, RNAs from NB4 and HL-60 cell lines have been artificially degraded by exposure to high temperature (100°C) for 20 minutes.

The degradation has been confirmed with Bioanalyzer instrument (Figure 35).

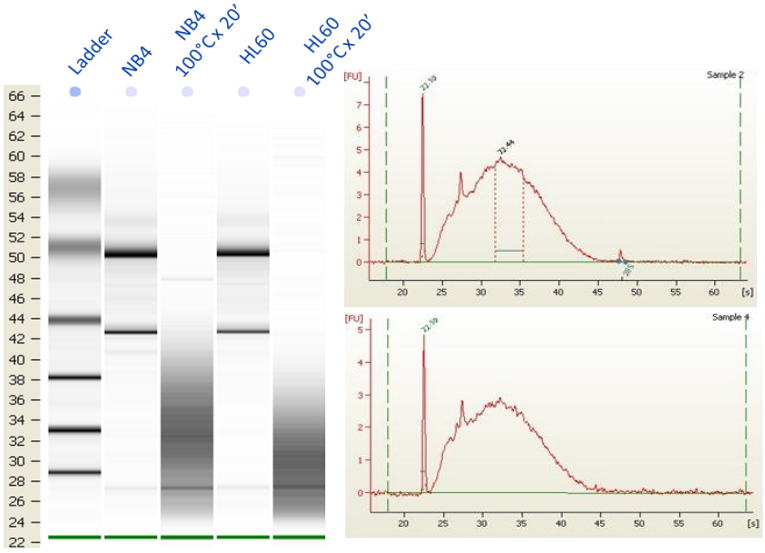

Figure 35. Results of the test with Bioanalyzer on the RNAs exposed to high temperature. The gel and the electropherograms show the high grade of degradation obtained.

On PML-RARA bcr1/3 assay, 20 replicates of NB4 dilutions 10^{-2} in HL-60, starting from degraded RNAs, have been correctly amplified, giving a delay in amplification of about 5 minutes (Table 20).

The assay proved to be robust also on the amplification of the internal control. On 20 replicates of degraded HL-60 RNA, the amplification of GUSβ showed only a 2 minutes delay (Table 20).

Sample	Replicates detected	AVG Tt (min)	
		Not degr.	Degr.
NB4	20/20	12.89	13.97
NB4 10^{-2}	20/20	16.5	21.67
HL-60	20/20	24.06	26.16

Table 20. Performance of PML-RARA bcr1/3 assay on degraded positive and negative samples.

On PML-RARA bcr2 assay, we could test the robustness of the internal control with 20 replicates of degraded HL-60. All of them have been correctly amplified with a delay of less than 3 minutes in GUSβ threshold times (Table 21).

Sample	Replicates detected	AVG Tt (min)	
		Not degr.	Degr.
HL-60	20/20	27.25	30.11

Table 21. Performance of PML-RARA bcr2 assay on degraded negative samples.

Also on real clinical samples degradation can frequently occur. 3 samples, negative for PML-RARA translocation, which showed a great level of degradation on the agarose gel have been tested by RT-Q-LAMP (Figure 33).

With the reference method RT-PCR those samples could not be amplified, but RT-Q-LAMP assays have been able to amplify them correctly in the internal control wavelength (Table 22).

Sample	Transcript type	PML-RARα bcr1/3 assay Tt (min)	PML-RARα bcr2 assay Tt (min)
BM1258/15	negative	26.45	33.45
UPN472	negative	24.43	32.03
UPN473	negative	27.93	34.67

Table 22. Amplification of 3 degraded clinical samples with PML-RARA assays

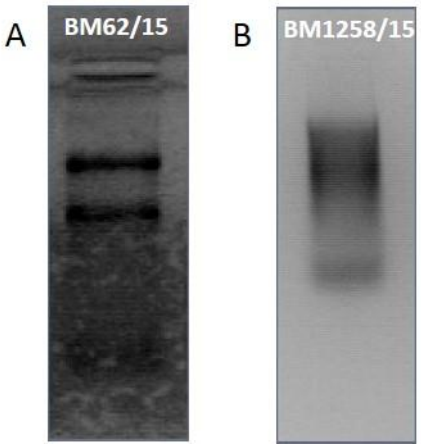

Figure 33. Agarose gel of samples BM62/15 (A) and BM1258/15 (B) showing respectively an example of good quality and degraded sample.

6.3 Robustness to low concentrated samples

Another issue that is frequently encountered by RT-PCR, in particular for treated leukemic patients, is the scarcity of RNA material extracted, especially from peripheral blood cells.

The starting amount of RNA for the PML-RARA assays is 500 ng/reaction, but both of them have been tested on lower amounts of RNA, on positive and negative samples and cell lines.

The results (Table 23) show for the triplex assay a good sensitivity on bcr1 down to 25 ng per reaction. At this concentration also the internal control is still detectable with only a minor delay in threshold times.

NB-4 10^{-2} concentration (ng/rx)	Replicates detected	AVG Tt (min)	SD	HL-60 concentration (ng/rx)	Replicates detected	AVG Tt (min)	SD
500	7/7	16.21	0.57	500	7/7	23.61	0.16
250	7/7	16.39	0.71	250	7/7	23.07	0.27
100	7/7	17.81	0.90	100	7/7	23.35	0.33
50	7/7	17.54	1.04	50	7/7	24.22	0.40
25	7/7	18.55	0.67	25	7/7	24.95	0.38

Table 23. Performances of PML-RARA bcr1/3 assay on different concentrations of NB-4 and HL-60 cell lines.

Also the duplex assay has demonstrated great robustness of the internal control down to 25 ng per reaction, even showing a speed up in the amplification of GUSβ (Table 25).

On duplex's sensitivity the impact of a lower amount of RNA has been evaluated on a clinical sample diluted down to 5 ng/µL and also further diluted 1:100. On the patient at the onset the PML-RARA bcr2 assay is able to detect even very low amounts of RNA, it starts to partially lose its sensitivity only when the sample is further diluted to 10^{-2} in HL-60 RNA (Table 24).

bcr2+ sample concentration (ng/rx)	Replicates detected	AVG Tt (min)	SD	bcr2+ 10^{-2} sample concentration (ng/rx)	Replicates detected	AVG Tt (min)	SD
500	2/2	15.43	0.08	500	7/7	25.51	1.46
250	7/7	15.89	0.43	250	7/7	26.05	1.89
100	7/7	16.03	0.23	100	11/12	25.78	2.45
50	7/7	15.98	0.13	50	9/13	25.94	4.37
25	7/7	16.46	0.77	25	11/16	25.25	5.13

Table 24. Performances of PML-RARA bcr2 assay on different concentrations of RNA on a bcr2 positive clinical sample.

HL-60 concentration (ng/rx)	Replicates detected	AVG Tt (min)	SD
500	3/3	26.89	0.40
250	6/6	24.80	0.58
100	6/6	22.71	0.57
50	10/10	23.53	0.50
25	10/10	23.69	0.66

Table 25. Performances of PML-RARA bcr2 assay on different concentrations of HL-60 RNA.

We could test the performance of the assays also on negative clinical samples in which the agarose gel showed a really low, close to zero, amount of RNA.

This particular sample has been rejected by the hospital laboratory because not adequate for the RT-PCR test. Conversely also in this case RT-Q-LAMP assays have easily detected the internal control (Table 26).

Sample	Transcript type	PML-RARα bcr1/3 assay Tt (min)	PML-RARα bcr2 assay Tt (min)
BM88/15	negative	24.53	24.64

Table 26. Results of PML-RARA assays on sample BM88/15.

DISCUSSION

Acute Promyelocitic Leukemia (APL) represents about 15-18% of all Acute Myeloid Leukemias[1]. In 99% of the patients it is characterized by the presence of the balanced translocation between chromosome 15 and chromosome 17, which gives rise to 3 different fusion transcripts (bcr1, bcr2 or bcr3), depending on the localization of the breakpoint on chromosome 15[16].

As a results of t(15;17), genes encoding for PML and RARα proteins fuse, producing the chimeric protein PML-RARα which induces a block of differentiation at the level of the promyelocytes, precursors of granulocytes[18].

Immature promyelocytes accumulate into bone marrow and peripheral blood, overexpressing many pro-coagulant factors, causing the activation of the clotting system, increasing fibrinolyitic activity and non-specific proteinase activity[11].

These disregulations are the principal elements that explain the severity of APL. At presentation about 80-90% of the patients show a severe hemorrhagic syndrome, especially in brain and lungs, which represents the main cause of early death[10]. About 20% of the patients die even before a definitive diagnosis can be formulated and the life-saving treatment administered[2].

Despite the aggressive nature of APL, the availability of specific drugs (ATRA and ATO) from the 1980s has radically changed the prognosis of APL from a rapidly fatal acute leukemia, to a highly curable disease[2]. The mechanism of action of ATRA and ATO is double: on one hand they relieve the block on transcription and on the other they promote the degradation of the chimeric protein PML- RARα, in both cases leading to the removal of the differentiation block[61, 62, 63].

The diagnosis of APL is based on the morphological features of the cells, after a smear blood analysis. However, the molecular confirmation is mandatory for the correct diagnosis. Nowadays the fastest method for the molecular confirmation is the RT-PCR, a laborious, time-consuming and multistep procedure since produces a not accurate result after at least 5 hours and without a clear identification of the specific PML-RARA translocation.

The fast progress of APL disease and the availability of highly effective therapy indicate the urgent need for a more performing molecular test, highly specific and rapid. Here in my thesis I describe and discuss a novel LAMP assay identified and further developed in DiaSorin.

Loop-mediated isothermal AMPlification (LAMP) is a powerful nucleic acids amplification technique, which has emerged as an easy-to-perform and rapid tool for molecular diagnostics applications in clinical routine[69].

LAMP amplifies DNA or RNA target with high specificity, efficiency and rapidity under isothermal conditions. It is fast, as the reaction is usually

completed in 60 minutes or less; it is relatively inexpensive thanks to the use of a strand displacement polymerase, avoiding the use of expensive thermalcycler instruments. All these peculiarities make LAMP intrinsically different from PCR and allow to achieve superior performances in those applications in which the unique characteristics of this method are magnified, as the diagnosis of leukemias.

Based on LAMP technology, this work described the development of an innovative molecular assay for the ultra-rapid diagnosis of APL and for the accurate discrimination of the 3 isoforms of PML-RARA translocation.

In DiaSorin the technology has been improved in two main characteristics, giving rise to RT-Q-LAMP: the coupling of retrotranscription and amplification activity in one enzyme, which allowed to start the reaction directly form RNA and in a single tube, and the use of fluorescent probes, which allowed the real time detection and discrimination of multiple targets.

Two separate assays have been developed: one for the detection and discrimination between bcr1 and bcr3 and a second one for the detection of bcr2. Both of them allow also the amplification of an internal control, a unique and remarkable characteristic, absent in RT-PCR, which is important for the validation of the negative results. The assays have been developed in a freeze-dried format that further improves the simplicity and rapidity of the technology.

The final freeze-dried formulations have been tested in terms of analytical performances. No false positives have been detected on over 500 replicates of negative RNAs and No Target Controls (NTCs). The analytical sensitivity has shown a high level of detection, reaching 10^{-3} dilution of NB4 RNA (carrying bcr1 mutation), 30 copies of bcr3 plasmid and 10 copies of bcr2 plasmid.

On 91 clinical samples no false positive or false negative results have been detected, comparing the results to RT-PCR or direct sequencing. Compared to PCR our assays are much more accurate in the identification of the correct transcript, with a 100% agreement with sequencing, while in uncertain cases PCR often failed the correct assignment of the isoform.

RT-Q-LAMP assays have demonstrated their extreme rapidity with a time-to-result of only 40 minutes, but this could even be shorter if we consider that all positive samples have been amplified in less than 20 minutes.

Even if the assays have been thought and designed for the detection of PML-RARA translocations in patients at the onset, that carry a high level of transcript, highly sensitive assays have been successfully developed. The sensitivity has been challenged diluting RNA from positive patients into negative RNA and both products have been able to detect the positive signal down to 10^{-3} dilutions.

Remarkably, the superiority of RT-Q-LAMP was even more evident on poor quality clinical samples, a condition that is often observed in the hospital laboratories[2]. In a variety of cases RT-Q-LAMP has been able to successfully amplify samples on which PCR had otherwise previously failed.

Those included highly degraded RNAs, samples with a very low concentration (< 10 ng/µl) and presence of inhibitors.

In conclusion, PML-RARA RT-Q-LAMP assays represent a new tool for the ultra-rapid molecular diagnosis of Acute Promyelocytic Leukemia, substantially improving the existing techniques and responding to clinical needs. Their peculiar rapidity, simplicity and reliability make them perfect for the fast molecular confirmation of APL, allowing in the shortest time the administration of the correct treatment to the patient. This superiority is even more remarkable on low quality samples, which cannot be analyzed throughout standard RT-PCR, but need to undergo a further extraction step for the collection of more or better material. In all the cases RT-Q-LAMP has been able to successfully amplify these samples, allowing a significant saving of time in clinical application.

After the industrialization phase, the assays developed during this PhD project will be launched on the market, being the first and unique molecular tests for the diagnosis of t(15;17) in less than 20 minutes.

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PUBLICATIONS AND POSTERS

Ultra rapid molecular detection of the PML-RARa fusion transcripts by Retro-Transcription Loop Mediated Amplification (RT-QLAMP) reaction on the semi-automatic Liaison IAM instrument.

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Semi-automatic ultrarapid detection of the PML-RARA fusion transcripts by retro-transcription loop mediated amplification (RT-LAMP) reaction on the Liason IAM instrument.

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Poster presented at EHA 18 Stockholm, 2013.

Retro-transcription loop mediated isothermal amplification (RT-Q-LAMP) as a rapid and robust molecular assay for the diagnostic detection of PML-RARA fusion transcripts.

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Poster presented at EHA 20 Vienna, 2015.

Simple, rapid and accurate molecular diagnosis of acute promyelocytic leukemia by loop mediated amplification technology.

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